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Index

No. 1

MANNING, A. M. and DAMASKE, E. E. (Geneva-Sampierdara) The Treatment of Terminal Metamorphosis of Chronic Granulocytic Leukemia with Cytarabine and Vincristine	1
FRANK, C. D. R. and COCHRAN, T. B. (Sutton) A Comparison of the Three in vivo Assays for Hemopoietic Stem Cells	9
KILGUS, E. (Duluth) Granulocyte Alkaline Phosphatase Activity: A Measure of the Immature Turn of Mature Marrow Neutrophils	19
RABIN, T., RABIN, R., and DOD, E. (Vareza) Spontaneous Placental Abruption in Multiple Gestations: A Preliminary Study	23
RABIN, S., RABIN, J., RABIN, G., and DAMASKE, E. (Geneva) Hemopoietic Stem Cell Assays (all 6 Grafts, And-Lysed) in an Human Tissue	30
HILGERT, C., DOD, E. R., ZIMMER, G. and RABIN, G. (Geneva) Overexpression of P-glycoprotein in the Human Myeloid Leukemia	35
HILGERT, M., RABIN, J., RABIN, E., and DAMASKE, E. (Geneva) Hemopoietic Stem Cell Assays in Patients with Acute Lymphoblastic Leukemia	41
FRANK, A. C., RABIN, J. G., LITVIN, V., and RABIN, E. (Geneva) Hemopoietic Stem Cell Assays in Patients with Chronic Granulocytic Leukemia	50
TANIGUCHI, T. (Miyagi) Morphology of Hemopoietic Stem Cells in Human Leukemia	56
(Index)	61

No. 2

FRANK, C. D. R., COCHRAN, T. B., and DAMASKE, E. E. (Geneva) Hemopoietic Stem Cell Assays in Patients with Chronic Granulocytic Leukemia	13
FRANK, C. D. R. and COCHRAN, T. B. (Sutton) Hemopoietic Stem Cell Assays in Patients with Chronic Granulocytic Leukemia	19
HILGERT, C., DOD, E. R., ZIMMER, G. and RABIN, G. (Geneva) Overexpression of P-glycoprotein in the Human Myeloid Leukemia	23
HILGERT, M., RABIN, J., RABIN, E., and DAMASKE, E. (Geneva) Hemopoietic Stem Cell Assays in Patients with Acute Lymphoblastic Leukemia	30
FRANK, A. C., RABIN, J. G., LITVIN, V., and RABIN, E. (Geneva) Hemopoietic Stem Cell Assays in Patients with Chronic Granulocytic Leukemia	35
TANIGUCHI, T. (Miyagi) Morphology of Hemopoietic Stem Cells in Human Leukemia	41

LEWIS, R. A. (Accra) Glucose-6-Phosphate Dehydrogenase Electrophoresis in Ghanaians with AA and SS Haemoglobin	103
MULLA, N. and CHORAK, L. (Kuwait) Haemoglobin C in Arabs in Kuwait	112
GIROLAMI, A., BRUNETTI, A., FIORETTI, D., and GRAVINA, E. (Padua) Congenital Thrombocytopathy (Platelet Factor 3 Defect) with Prolonged Bleeding Time but Normal Platelet Adhesiveness and Aggregation	116
YAMAK, B., ÖZSOYLU, S., ALTAY, C., HİÇSÖNMEZ, G., and SAY, B. (Baltimore) Hereditary Persistence of Fetal Hemoglobin and β -Thalassemia in a Turkish Child	124

No. 3

DOUGLAS S. D., COHEN, G., KÖNIG, E., and BRITTINGER, G. (New York, N.Y.) Ultrastructural Features of Phytohemagglutinin and Concanavalin A - Responsive Lymphocytes in Chronic Lymphocytic Leukemia	129
BROTHMAN, J. (Aarhus) Diisopropylfluorophosphate Uptake by Granulocytopenic Cells in Chronic Myeloid Leukemia and in Normal Individuals	143
PERTAKIS, A., PANTISAS, G., and ANGELLOPOULOS, B. (Athens) Serum Haemopexin Concentration in Patients with Various Haemoglobinopathies: Effect of Splenectomy	149
FALTER, MARIA L., ROBINSON, MARGARET G., OK SOON KIM, SUAT CHENG GO, and TAUBKIN, S. P. (Brooklyn, N.Y.) Splenic Function and Infection in Sickle Cell Anemia	154
GIRAUDO CONTRA, L. V., RUMI, L., COLMERAUT, M. E. M., and DOÑE PASQUALI, C. (Buenos Aires) Ribonucleic Acid and Phytohemagglutinin on Rat Leukocyte Cultures within Diffusion Chambers	162
CSEJLÁTI, J. and TÓTH, S. (Szeged) Study of the Effect of Actinomycin D on the Thrombocytopoiesis of Mice Using ^{75}Se -Labelled Methionine	165
MINSTER, H. and TRAU, F. (Erfurt) Unreife zellige Panmyelose	174
ÖZSOYLU, S., HİÇSÖNMEZ, G., and ALTAY, C. (Ankara) Hemoglobin H β -Thalassemia	184
Libri	191

No. 4

SHICK, B. and KISSLING, M. (Leiden) Studies on Bone Marrow Transplantation in Experimental ^{32}P Induced Aplastic Anemia After Conditioning with Anti-lymphocyte Serum	193
RAISER, H., HÖCKER, P., PITTERMANN, E. and MOSER, K. (Wien) Säulenchromatographische Anreicherung von DNA Polymerase Aktivität bei Leukämie	200

- HEZARD, B., FAHMY, R. et HERRMANN, G. (Bordeaux) Le syndrome de Rich et Ravnitzky de quatre observations et essai de détermination 215
- HIRS, A. und KLEINER, U. (Wien) Untersuchungen über den Einfluß des Cystophosphorsäure auf Enzyme der Megakaryocyten 217
- BRIMMING, L. and BARNAT, F. (Louvain-la-Neuve) Some Aspects of Leucocyte Behaviour in Haemophilia 223
- CHIRIAU, A., SCORZA, P., BRUNETTE, A., MONTAGNE, C., and SARTRE, G. (Rennes) Congenital Hypofibrinogenemia (Factor VII Deficiency). A Review of Two Cases Bearing on Two Different Kinetics 229
- ONEL, D., LUTTEN, S. B. and LUTTEN, O. W. (Hamburg) Platelet Defect in a Case of Ehlers-Danlos Syndrome 231
- HILKE, D. und ALBRECHT, H. (Frankfurt) Neuartige Erkrankung des Ergankens peripherer Lymphocyten bei Virusinfekt 243
- CHAMBERS, J. D., KIM, M. C., and KATZ, J. (St. Louis) Thrombocytosis in Hest Stroke 249

No 5

- PARSONS, O. L., GASSETT, VERA, and GAYNOR, F. (Toronto) Mechanism of Action of Aspirin on the Cell Cycle and Growth in Acute Lymphoblastic Leukemia 255
- LEWIS, JAMES A. (Ann Arbor, Mich.) The Effect of Aspirin on DNA and RNA Synthesis by Lymphoblasts of Acute Lymphoblastic Leukemia 257
- ARITA, T., IACONA, V., FERRILLI, M., and ORIO, MARIA VERA (Rome) Lymphocyte Surface Materials in Lymphoproliferative Disorders 259
- MILSTEIN, T., HANSEN, P., PARSIA, R. L., and MURRAY, G. (Turku) Bearing of Tissue Acid to Serum Proteins. II. The Effect of Dehydration on Tissue Water and of Various Diseases 264
- TURNER, F. M., SWEET, J. A., and FRANKS, T. L. (Pittsburgh) The Persistence of Anticardiolipins and Antibodies in Haemophilia 273
- SHANKS, PETER (Durham) Blood Counts and Tissue Metabolism in a Child with Haemophilia 279
- ORVILLE, R. and O'NEILL, L. (Aberdeen) Acquired Factor IX Deficiency. A Review of Ten Cases 285
- LEWIS, A., FRIEDMAN, R. B., and COHEN, B. (Abingdon) Haemophilus D Protein in Cerebral Lesions and its Relation to Haemophilus 293

No. 6

- LEWIS, C., JENNINGS, J., BROWN, M., and JENNINGS, C. (Aberdeen) The Value of Laboratory Tests in the Diagnosis of Patients with Haemophilus 301

MEURET, G., LOWKA, A., BRANDI, E. T. and KALKOFF, A. W. (Freiburg) Erythrodermia Mycosis fungoides, Skin Reticulosis - Autonomous Disorders of the Monocytopoietic Macrophage System?	329
MÖLLER, D., LAUTERBACH, H., POULLEON, H. G. und HAHN, E. (Tübingen) Synthese von Hämoglobin, RNS und Proteinen in der normalen Erythropoese	340
ZACCARIA, A., RICCI, P., BACCARANI, M., and TURR, S. (Bologna) Chromosome Studies in Paroxysmal Nocturnal Haemoglobinuria	350
WEINSTEIN, B. I. DE, WHITE, J. M., WILTSHIRE, B. G. and LEHMANN, H. (Cambridge) A New Unstable Haemoglobin Hb Buenos Aires, $\beta 85$ (F1) Phe \rightarrow Ser	357
Varia	364
Index rerum ad Vol. 50	365
Index autorum ad Vol. 50	376

ly tested with success in acute lymphoblastic leukaemia (ALL). Out of 30 patients treated with 2 mg m² of VCR weekly and 60 mg m² of Pred daily 9 (30%) achieved a complete or partial remission and 6 of the 9 had a complete haematologic remission with return of the marrow morphology to normocellularity or to the granulocytic hyperplasia characteristic of the myelocytic phase of CGL. In addition in 5 of these patients the aneuploid line that characterized their blastic transformation completely disappeared with a return to the chromosomal constitution of the chronic phase of the disease (46 chromosomes Ph⁺ positive). The mean duration of the first remission in the complete remitters was 5 months. Furthermore in one patient having presented with the clinical and haematologic features of acute myeloblastic leukaemia but with an aneuploid Ph⁺ positive blast cell population a prolonged remission was obtained resulting in a 28 month survival with the features of CGL, while progressive refractoriness to chemotherapy was associated with the reappearance of the same aneuploid blast cell line and by the emergence of an additional aneuploid clone [7].

Since we too have been using much the same combination for the last three years we should like to report our own experiences which besides confirming the efficacy of this simple treatment have enabled us to observe a marked difference between the two fundamental cytologic types of metamorphosis.

Clinical Observations

A VCR-corticosteroid (generally dexamethasone or prednisolone) combination practically superimposable to the schedule of CASTILLOS *et al* [6] has been employed for the treatment of metamorphosis of CGL in our Department for the last 3 years. However no single dose of VCR superior to 2.0 mg was ever given in order to avoid severe neurotoxicity.

The diagnosis of metamorphosis was made according to the generally accepted criteria [4-6, 12, 19, 22, 25] however a distinction was made between genuine *Elastic transformation* of bone marrow and blood (fig 1) composed of basophilic agranular undifferentiated cells corresponding to the so-called promyeloblastic type of MATTEI *et al* [19] and what we prefer to call the granular type of metamorphosis that is a generally gradual (more rarely abrupt) augmentation of immature granulocytic cells always characterized by the abundance of azurophilic granules in the cytoplasm when stained with the Romanovsky's mixtures.

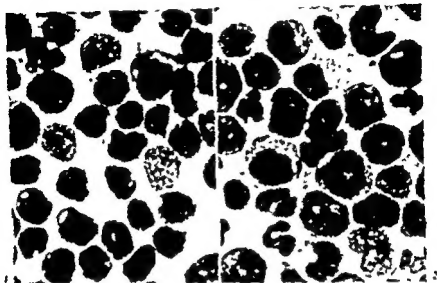


Fig. 1. Typical agranular blastosis as terminal metamorphosis of CLL.

Fig. 2. The appearance of granular metamorphosis, which is different both from the former type and from the picture of blast, uncomplicated CLL.

(Fig. 2). At times, the number and uniform appearance of such cells mimicked the appearance of acute promyelocytic leukaemia (APL), as it has been already remarked [4]. However, in our experience, Auer rods are abundant in APL, but always absent in metamorphosis. In this category, 2 cases with marked hyperleukocytosis ($>40 \times 10^9/l$) were found, although thrombocytopenia had not been prominent all along the myelocytic phase.

Although mixed pictures could be observed in a few cases, both for the sake of simplicity and in order to obtain a better evaluation of the therapeutic results, all cases were divided in 2 categories according to the predominant feature, that is an 'agranular' and a 'granular' type. Both were treated with the VCR and combination for periods never exceeding 6 weeks.

The results are shown in Table 1 in which the well-known criteria for the acute leukaemia have been adopted for the evaluation of complete and incomplete remissions.

Complete and incomplete remissions were subsequently treated with a combination of 6-MP (12.5 mg/kg/day) and MTX (15 mg/m^2).

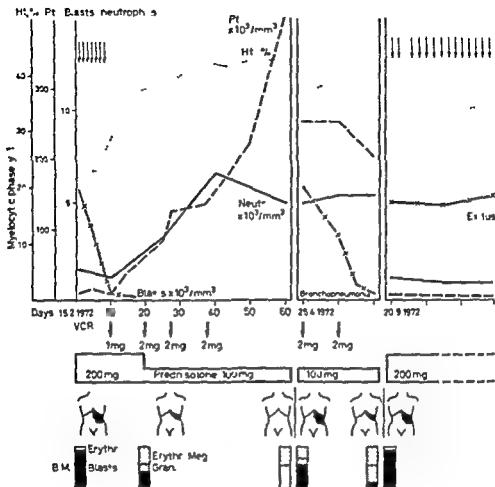


Fig 5 Non granular blastic metamorphosis of CGL.

ized CANELLOS *et al* [6] have presented evidence indicating that the hypodiploid cell lines are significantly more sensitive to this combination, and have speculated that they may contain less microtubular spindle protein, and be accordingly more sensitive to the antimicrotubular properties of VCR. However reduction of chromatin material as in hypodiploidy does not appear to be necessarily coupled with a diminution of microtubular protein which should be ascertained separately. In addition the prompt and dramatic oncolytic effect of corticosteroids on these cells cannot be explained in terms of microtubular damage: steroids notoriously attack lymphoblasts also in the resting stage, besides arresting them in G_1 [11] a mechanism which has been recently

shown to be operative also for VCR [21]. It is conceivable that the sensitivity of these cells may result from a combination of their proliferative state and intrinsic metabolic properties.

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A Comparison of the Three *in vitro* Assays for Haemopoietic Stem Cells

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Abstract The effect of a proliferation-independent (nitrogen mustard) and 4 proliferation-dependent (methotrexate, vinblastine, cyclophosphamide and 5-fluorouracil) cytotoxic agents has been investigated concurrently on the 3 *in vitro* assays (colony forming units, erythroid repopulating ability and granulocytic repopulating ability) for haemopoietic stem cells. These preliminary results show that the proliferation-dependent agents produce different responses in the 3 assays consistent with the presence of 3 different cell populations proliferating at different rates. The findings tend to support published hypotheses on the existence of 3 different stem cell populations (possibly 'committed' stem cells).

Key Words

Cytotoxic agents
Erythroid repopulation
Granulocytic repopulation
Haemopoietin
Proliferation rates
Spleen colony formation

Several *in vivo* assays have been used to estimate the proliferation of haemopoietic stem cells. The response to erythropoietin in polycythaemic mice [10] is now known to measure a cell population (the erythropoietin responsive cells) distinct from the haemopoietic stem cells [3, 4, 18, 23]. However, there are 3 other assays which are still considered to measure stem cells. The spleen colony technique [22], the erythroid repopulating ability technique based on the ability of transplanted bone marrow to restore erythropoiesis, as measured by ^{59}Fe uptake, in bone marrow-depleted recipients [15, 24] and the granulocytic repopulating ability technique in which the granulopoietic capacity of transplanted bone marrow is measured by the response of the peripheral blood neutrophils to endotoxin [5, 12].

If the concept of a single type of bone marrow 'stem' cell which is multipotential (i.e. can produce differentiated progeny of all haemopoietic

cell lines) is valid, it would be expected that for a given pre-treatment to the donor animal, the same result would be obtained from each of the 3 assays, provided that the treatment did not influence the differentiation pathways of the surviving multipotential 'stem' cells. The evidence so far available, however, indicates that the erythroid and granulocytic repopulating abilities, in mice, respond in different ways to X-irradiation and cyclophosphamide [13, 14] and, in rats, to cyclophosphamide and repeated injections of methotrexate [7, 8].

It was felt that the best way of investigating the relationship between these precursor cell assays was to measure all three, *concurrently*, using the same sample of bone marrow. The donor mice were treated with single doses of various cytotoxic drugs taken from each group of the classification scheme for cytotoxic agents, as established by BRUCE *et al* [5] on the basis of target cell proliferation rates. Class I agents (as exemplified by nitrogen mustard) show exponential dose/response graphs with both slowly and rapidly proliferating cells having a similar sensitivity. Class II agents (phase specific, e.g., methotrexate and vinblastine) act over one phase of the cell cycle. The dose/response graphs show plateaus because only those cells in the sensitive phase of the cycle are killed. The plateau is at a lower survival for rapidly proliferating cells than for slowly proliferating cells since more cells pass through the sensitive phase during the time for which the drug is active. Class III agents (cycle-specific, e.g., cyclophosphamide and 5-fluorouracil) act on proliferating cells throughout the cell cycle. The dose response graphs are exponential but the graph for rapidly proliferating cells is steeper than for slowly proliferating cells.

The following results represent our preliminary findings in which the response in the 3 *in vivo* assays for haemopoietic stem cells have been compared. Nitrogen mustard (HN2), methotrexate (MTX) and cyclophosphamide (CY) were studied in some detail. Pilot experiments were carried out using vinblastine (VB) and 5-fluorouracil (5-FU) to determine whether the responses observed after MTX and CY were specific for MTX and CY or were characteristic of class II (MTX and VB) and class III (CY and 5-FU) agents generally.

Materials and Methods

Chemicals. Nitrogen mustard (mustine hydrochloride) was obtained from Boots Pure Drug Co. Ltd., Nottingham; methotrexate from Cyanamide International, Pearl River, N.Y., vinblastine sulphate (Velbe[®]) from Eli Lilly & Co. Ltd., Basing

stoke, cyclophosphamide (Endosana) from Ward Blenkinsop & Co Ltd., London, and 5 fluorouracil from Roche Products Ltd., Welwyn Garden City

All these agents were dissolved in saline and the solutions prepared immediately before injection. The chemicals were administered intravenously, to the donor mice 24 h before the marrow was taken for assay

Animals C57Bl/6 mice, aged approximately 12 weeks at the start of experimentation, were used throughout. Between 3 and 12 mice served as bone marrow cell donors in each group. The bone marrow from these animals was assayed simultaneously for colony forming units, erythroid and granulocytic repopulating abilities

Approximately 4 h before receiving the bone marrow, the recipients for all 3 assays were exposed to 700 rad ^{60}Co -rays (dose rate 45 rad/min). This dose of irradiation was sufficient to reduce the mean endogenous colonies to less than one per spleen

At least 6 recipients per group were used for the spleen colony and erythroid repopulating ability assays and 7 for the granulocytic repopulating ability assay

Varying numbers of treated bone marrow cells were transplanted such that the responses in all 3 assays were always on the linear part of the respective dose response graphs

Spleen colonies (CFU) The method for the assay of colony forming units (CFU) was basically that described by TUN and MCCUSKER [22]. Eight days after bone marrow transplantation, the recipient animals were killed and their spleens excised and fixed in Bouin's solution. All macroscopic colonies on the flat surface of the spleens were counted

Erythroid repopulating ability (EPA) This technique has been fully described earlier [15, 24]. The mouse was injected into the recipient mice 7 days after bone marrow transplantation and the uptake into the blood determined 28 h later. Blood volume data were used to express isotope uptake in terms of total activity in the blood animal according to the previously reported method [24]

Granulocyte repopulating ability (GRA) The peripheral blood granulocyte response to endotoxin was assayed 9 days after bone marrow transplantation by a modification of the method of HIRSHMAN and GRANT [12]. $10 \mu\text{g}$ *S. typhosa* endotoxin (Difco Chemical Co.) were injected intraperitoneally into each recipient animal and 8 h later. At the time of maximum granulocyte response [21], blood samples were taken and the concentration of granulocytes in the blood determined from total leukocyte counts (measured with haemocytometers) and peripheral blood leukocyte differentials determined from blood smears.

HIRSHMAN *et al.* [13] have shown that the GRA of irradiated mouse bone marrow is not significantly different when determined 9 or 31 days after transplantation and in fact the GRA of marrow treated with cytotoxic drugs is independent of the time after transplantation [6]. Therefore it was considered that a 9-day assay of GRA would be suitable for these preliminary studies.

Calculation of results In all 3 assays the results are expressed relative to one control. The means and standard errors were calculated from the individual results in each group of recipients. The means \pm standard errors of the experimental groups are expressed as fractions of the relevant control value \pm its standard error. Thus, the points on the following figures represent the surviving fraction of control \pm an error which includes the variation in the control group.

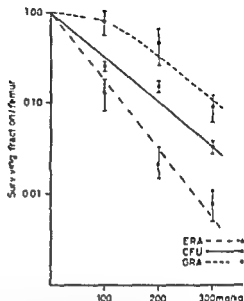
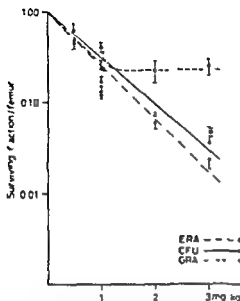


Fig 1 Erythroid repopulating ability (ERA) colony forming units (CFU), and granulocytic repopulating ability (GRA) per femur of mouse bone marrow 24 h after nitrogen mustard. Vertical bars indicate \pm one standard of the mean. For clarity, the top or bottom error bars have been omitted from certain points. Mean results of 3 experiments.

Fig 2 Erythroid repopulating ability (ERA), colony forming units (CFU), and granulocytic repopulating ability (GRA) per femur of mouse bone marrow 24 h after cyclophosphamide. Vertical bars indicate \pm one standard error of the mean. Mean results of 3 experiments.

Results

Nitrogen mustard had similar effects on the ERA and CFU both decreasing exponentially with dose as expected for a class I agent. However, the GRA decreased to a plateau at the level of approximately 20% survival (fig 1). Since after irradiation (another class I agent), it has been shown [12] that all 3 assays show an exponential decline with increasing dose, the plateau in GRA after nitrogen mustard would seem specific to this agent rather than class I agents generally.

Single doses of *methotrexate* produced only approximately 50% depression in survival for all 3 assays. All doses used appeared to be on the plateaus in the dose response curves and there was possibly a slightly greater survival of GRA than of ERA and CFU (table I).

Table I

Dose of methotrexate mg/kg	Surviving fractions/centur of		
	ERA	CFU	GRA
40	0.55 ± 0.06	0.51 ± 0.04	0.84 ± 0.23
100	0.68 ± 0.10	0.47 ± 0.06	0.81 ± 0.23
200	0.65 ± 0.18	0.54 ± 0.04	0.72 ± 0.27

Erythroid repopulating ability (ERA), colony forming units (CFU), and granulocytic repopulating ability (GRA) per femur (\pm one standard error of the mean) 24 h after methotrexate. Mean results of 3 separate experiments.

Table II

Dose of vincristine mg/kg	Surviving fractions/centur of		
	ERA	CFU	GRA
0.5	0.04 ± 0.01	0.10 ± 0.01	0.24 ± 0.07
1.0	0.05 ± 0.01	0.04 ± 0.01	0.12 ± 0.02

Erythroid repopulating ability (ERA), colony forming units (CFU), and granulocytic repopulating ability (GRA) per femur (\pm one standard error of the mean) 24 h after vincristine sulphate. Mean results of 2 experiments.

The results with vincristine (table II) were essentially similar to those obtained following methotrexate (table I). In view of the findings of *Stuart et al.* [20] it was not possible to be certain that the doses used corresponded to the plateau regions on the dose response curves as demonstrated for CFU by *Blaxter et al.* [5] using similar doses.

The survival in all 3 assays after cyclophosphamide decreased exponentially with increasing dose but with different slopes for each assay (fig. 2). There was some indication of an initial shoulder on the GRA curve. The CFU curve lay between those obtained for GRA and ERA. Using similar doses, *Hallman et al.* [14] observed a reduction in GRA to a plateau level of 30% survival following cyclophosphamide which contrasts with the exponential decrease shown in figure 2. A direct comparison

Table III

Dose of 5 FU mg/kg	Surviving fractions femur of		
	ERA	CFU	GRA
40.0	0.14 \pm 0.02	0.19 \pm 0.02	0.62 \pm 0.14
80.0	0.11 \pm 0.002	0.09 \pm 0.01	0.19 \pm 0.05
100.0	0.009 \pm 0.003	0.06 \pm 0.01	0.09 \pm 0.01

Erythroid repopulating ability (ERA) colony forming units (CFU) and granulocytic repopulating ability (GRA) per femur (\pm one standard error of the mean) 24 h after 5 fluorouracil. Mean results of 3 experiments.

son between the results of HELLMAN *et al* [14] and those now reported is difficult, since in HELLMAN's study cyclophosphamide produced only a small depression of CFU. Other reports [5, 26] have shown cyclophosphamide to have a marked effect on CFU similar to that now reported.

Preliminary results with 5-fluorouracil (table III) indicated that the type of response was similar to that seen after cyclophosphamide (fig 2) and suggest that the different responses in these 3 assays were typical of class III agents.

Discussion

It has been shown that erythroid and granulocytic repopulating abilities and spleen colony-forming units each respond in different ways to treatment with certain cytotoxic agents. The reduction of granulocytic repopulating ability to a plateau following nitrogen mustard (fig 1) was not found with the other 2 assays. The cycle-specific agents, cyclophosphamide (fig 3) and 5 fluorouracil (table III) produced exponential decreases in survival but with different slopes for the 3 assays and there appeared to be initial shoulders on the GRA curves. The results also suggested that the phase-specific agents methotrexate (table I) and vinblastine (table II) had less effect on GRA than on CFU and ERA.

Both the CFU assay [9] and the cells measured with the repopulating assays [3, 25] measure populations of primitive haemopoietic cells with considerable capacity for proliferation and which have distinct kinetic characteristics from the more mature, morphologically recognisable, haemopoietic cells. All 3 *in vivo* stem cell assays are dependent, probably to

varying degrees, on the proliferative capacity of the viable, transplanted stem cells. This ability may or may not be altered by treatment of the donors with cytotoxic agents, although it would depend on an induced defect in differentiation and/or maturation being transmitted through many cell divisions [9]. Should a change in the output of mature cells stem cell occur, it might be expected to influence the CFU and repopulating assays in different ways. Since the CFU assay depends on the scoring of clones of the progeny of individual stem cells in one organ only (i.e. the spleen), changes in cell output from the stem cells could possibly result in a reduction in colony size without, necessarily, in colony number. Changes in cell production, however, might contribute to changes in the repopulating assays which depend on the integrated output of mature differentiated cells derived from all the transplanted repopulating cells. If all 4 proliferation-dependent cytotoxic agents (MTX, VB, CY and 5-FU) produced a similar block in differentiation and/or maturation specific to the same haematological cell line then the different survival curves, reported above, might be expected.

The type of mature cell produced may depend on the organ (e.g. bone marrow or spleen) in which the transplanted stem cell lodges and proliferates [27]. It seems unlikely, however, that drug treatment of the donors would lead to a redistribution of surviving stem cells in the recipients sufficient to account for the different responses of the 3 assays.

The CFU, IRA and GRA assays may measure distinct cell populations which respond in different ways to treatment with proliferation-dependent cytotoxic agents. With the exception of the GRA curve after nitrogen mustard (see above) the shapes of the dose response curves for the 3 assays are as expected for three different cell populations proliferating at different rates [5]. These results, therefore, favour the existence of different populations of stem cells, and would be consistent with the considerable amount of evidence from other types of investigation [1, 2, 7, 8, 17-18] which have suggested the existence of different populations of repopulating cells committed to separate lines of haemopoietic differentiation. The present studies with proliferation-dependent cytotoxic agents then lead to the conclusion that the erythroid repopulating cells proliferate rather more quickly and the granulocytic repopulating cells somewhat unexpectedly more slowly than the CFU. Results with the *in vitro* and *in vivo* neutralisation technique in a different strain of mice to those

* D. S. C. D. R. and C. M. T. H. reported observations.

used in the present report suggest similar differences in the proliferation rate of the cells measured with these 3 assays

Further studies need to investigate the response in the CFU, ERA and GRA compartments with time after administration of the cytotoxic agents and to determine any alteration in the seeding efficiency of the CFU or in colony size. Also a comparison of the responses in the 3 *in vivo* assays for stem cells, particularly the granulocytic repopulating ability, with those obtained using the *in vitro* bone marrow cloning technique will be of considerable interest since the granulocytic repopulating cells appear to be proliferating more slowly than the CFU while the available evidence [11, 16-19] suggests that the cells producing granulocytic colonies *in vitro* are proliferating more rapidly than the CFU.

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Granulocyte Alkaline Phosphatase Activity: A Measure of the Emergence Time of Mature Marrow Neutrophils?

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Abstract Early release of mature bone marrow granulocytes either with 'empty' stores or with exaggerated turnover rate of the marrow store furnishes more alkaline phosphatase positive neutrophils, whereas release of already stored cells furnishes more alkaline phosphatase negative cells. Thus, providing other factors, such as release of splenic or marginal granulocytes, do not disturb the condition, neutrophil alkaline phosphatase activity appears to reflect the bone marrow retention of candidate cells. The biphasic response to adrenal steroids could be explained by this hypothesis.

Key Words
Agranulocytosis
Bone marrow aplasia
Cell release
from bone marrow
Granulocytopoiesis
Leukocyte
alkaline phosphatase
Leukocyte kinetics
Pancytopenia

A relatively abundant amount of works deals with the explanation of granulocyte alkaline phosphatase positivity and negativity. Owing to the fact that, in general, enzyme activity diminishes with cell age, the decisive role of cell life span was considered [1], but it was subsequently suggested that granulocytes released from the bone marrow, either as a result of some injury, or of external stimulation, such as endotoxin or ethoxholanoline, are alkaline phosphatase negative [2-4] and positivity develops subsequently in the vascular bed. In this sense, a shift to alkaline phosphatase negative cells would mean something like reticulocytosis. Quite recently, Peterson and Hamner [5] theorized that low granulocyte alkaline phosphatase activity in chronic granulocytic leukaemia (CGL) is a manifestation of incomplete maturation of cells, and the rise of activity in so-called blastic crises of CGL might be caused by an influx of fully matured granulocytes whose origin is the spleen rather than the bone marrow. Interestingly, a rapid transit through the marrow ma-

turation compartment was mentioned as a possible explanation for the low enzyme activity of CGL granulocytes.¹

I now report on 4 cases of drug induced granulocytopenia in which the alkaline phosphatase activity of granulocytes was investigated, both in bone marrow and peripheral blood smears, at the earliest phase of recovery of granulocytopoiesis. Cases 1 and 2 suffered from pancytopenia, whereas cases 3 and 4 had overwhelming granulocytopenia. Five cases of chronic idiopathic pancytopenia with hypocellular marrow (controlled by trephine biopsy) have been included, too.

Method

The modified Laplow method described by HAYHOE and QUAGLINO [6] was applied to demonstrate alkaline phosphatase and activities were scored, accordingly, from 0 to 4. The upper score limit regarded as non pathological in our laboratory, is 150.

Case Histories

Case 1 A 57 year old man with marginal erythrocytosis has been erroneously diagnosed as suffering from polycythemia vera in a hospital and ingested daily 750 mg dibromomannitol (DBM) for 3 weeks (*) without adequate blood cell control. He was admitted to the clinic 18 days after the withdrawal of DBM. He suffered from septicæmia, his granulocyte count was zero, and only a few hundred small lymphocytes were present in peripheral blood smears. The first bone marrow samples were severely hypocellular, contained a few cell types only, small, and occasional medium sized lymphocytes, plasma cells, macrophages and a somewhat homogenous early progenitor cell population.

The first granulocytes (200 μ l) appeared in the circulating blood as soon as 2 days after the above mentioned bone marrow investigation - which disclosed virtual absence of recognizable granulocyte precursors (1/1 000 cells) - indicating an extremely rapid whole marrow transition time in this emergency situation and suggesting immediate release from empty reservoirs.² Along with this the representations of the potentially alkaline phosphatase positive granulocytes increased from 1/1 000 cells to 50/1 000 cells during a 72 hour period. At this time practically all bands and segmented neutrophils showed remarkable alkaline phosphatase positivity (table I). However at least 2 factors influenced this result: sepsis and prednisolone administration. Two years after recovery, the patient is free of any haematological pathology.

* Many other workers have made significant contributions to this problem but no attempt was made to review the literature.

² Otherwise some atypical origin of these granulocytes should be presumed.

Table 1 Intensity of granulocyte alkaline phosphatase (scores from 0 to 4) in % early at the recovery of injured granulocytopenous

Case No	Circulating blood					Bone marrow				
	0	1	2	3	4	0	1	2	3	4
1	-	20	30	30	12	6	31	38	18	7
2 ¹	-	-	2	10	88	-	-	-	² / ₁₀	³ / ₁₀
3	-	-	5	41	54	-	-	11	24	61
4 ²	5	3	8	43	43	6	7	23	49	14

¹ Only 50 peripheral granulocytes were calculated in this case and 10 mature granulocytes could be discovered in the marrow

² Metamyelocytes, or even myelocytes were positive in the marrow

Case 2 A 39 year-old woman, another patient treated by dibromomannitol on the base of unproven polycythaemia vera ingested 6.5-10.0 (?) g of the drug during a 9-day period and was admitted to the clinic in a poor condition with 200 WBC μ l 23 days after withdrawal of DBM. The bone marrow exhibited predominant fatty areas along with groups of mainly lymphocytes and plasma cells, but occasional progranulocytes were present as well as mature eosinophils. Mature neutrophils were, however, virtually absent.

Although clinical signs were not overwhelming, the patient was placed in a pathogen arm room, got wide spectrum antibiotics, but prednisolone was not given. Table 1 shows the phosphatase scores at the day of the death of the patient, the 2nd day of our observation. She had hypertensive cardiovascular disease with congestive splenomegaly and apoplexy terminated her life.

Case 3 A 64 year-old diabetic woman had severe agranulocytosis after about 5 weeks of treatment by the oral antidiabetic drug Invenol®. Her WBC count was 250 μ l and her bone marrow differential was quite similar to that of case 2. With drawing the harmful drug, she became free of fever in the pathogen arm room already 2 days before the bone marrow investigation, and did not receive prednisolone in this period. The peripheral WBC count rose to 3000 μ l within 2 days, 15% of them being granulocytes. Phosphatase values at this early period of recovery, i.e. the 2nd day of sudden clinical and haematological improvement are represented on table 1.

Case 4 A 72 year-old man with haemiparesis and oarcomatosis developed a clinical picture where granulocytopenia caused by cloxacillin and cyclophosphamide therapy. The number of granulocyte count dropped to 400 μ l with about 20 neutrophils, but there was no fever and no recognized systemic infection, the bone marrow infiltration was only moderately enhanced, and every member of the granulocyte system was present with a shift to the left. Prednisolone was not given, even before. At the earliest phase of recognized improvement of granulocyte count (table 1, 2) at the beginning of an apparently spontaneous recovery of

granulocytopenia without any therapeutic measure alkaline phosphatase activities were investigated (table I)

Cases 5-8 Our last 5 cases of true chronic idiopathic aplastic pancytopenia exhibiting severe bone marrow hypocellularity as controlled by trephine biopsy, showed granulocyte alkaline phosphatase scores over 250 in peripheral smears, before any recent therapy and in the absence of clinically recognizable systemic infection in at least 3 cases

Discussion

Whatever would be the correct explanation for these and all available adequate clinical observations, the above trials appear to demonstrate that in different kinds of severe granulocytopenia with or without other cytopenia and with or without recovery, a great part of mature granulocytes exhibit increased alkaline phosphatase activities at the early days of successful or unsuccessful attempts to recover from granulocytopenia in the marrow as well as in the peripheral blood. This means, that in these emergency situations in which, with one exception, marrow granulocyte reserves were absent, and at least at the beginning of recovery granulocyte production rate should be greatly increased along with an apparently immediate release of granulocytes from the marrow. Granulocytic alkaline phosphatase activities are already significantly increased.

The eventual role of prednisolone causing high phosphatase scores can be excluded (cases 2 and 4) and that of systemic infection does not appear to be decisive (case 4). Chronic cases of true aplastic pancytopenia with not infrequent absence of recognized systemic infection also speak against the exclusive role of systemic infectious states. Table II attempts to give a superficial information about the state of certain conditions related to changes in granulocyte alkaline phosphatase activity.

There are, of course, other factors which may influence the enzyme activity of the circulating granulocyte, such as their location (e.g., marginal pool, splenic pool) and their elimination rate, and the significance of these factors must be considered in every single case.

If we consider granulocyte alkaline phosphatase activity to indicate a kinetic parameter, its differential diagnostic usefulness would be less than generally supposed. In fact, although the majority of our 66 chronic granulocytic leukaemia cases exhibited quite low numbers of alkaline phosphatase positive cells, normal or elevated counts occurred at each time period of the process (46% in its 1st year, 25% in years 2, 3, 4-5).

Table II Type of released mature marrow granulocytes related to their alkaline phosphatase activity¹

Condition	Mature granulocyte reserves in the marrow	Type of released mature marrow granulocytes	Granulocyte alkaline phosphatase activity, direction of change towards
Normal	steady state with storage	steady state with storage	
Normal, stimulated e.g. endotoxin subsequent phase	steady state with storage reduced ²	stored newly formed	negativity positivity
Steroid administration, early phase	present	stored	negativity
subsequent phase	reduced	newly formed	positivity
Surgery	present	stored	negativity
Aplasia, pancytopenia	reduced or absent	newly formed	positivity
Recovery from aplastic pan- cytopenia or agranulocytosis	reduced or absent	newly formed	positivity
Skeletal hyperplasia	reduced ²	newly formed	positivity
Advanced myelofibrosis	reduced	newly formed	positivity
Chronic granulocytic leukaemia	increased	stored	negativity
Acute leukaemia	reduced ²	stored ²	negativity
Polycythaemia vera	increased	newly formed ²	positivity

¹ The uncertainty of certain statements is signified by question marks

and 31% in years 6 and 6+) despite the exclusion of the periods of blastic transformation from the material.³

In 5 out of 8 determinations performed of 3 chronic granulocytic leukaemia patients who were inadvertently rendered aplastic by chemotherapy Rosvick *et al.* [7] found marked increases in enzyme level and in at least one patient a bone marrow chromosome preparation, performed at a time when the enzyme level was markedly increased, showed the Philadelphia chromosome in all evaluable clones. Further, leukocyte alkaline phosphatase is able to return in chronic granulocytic leukaemia mar-

² These results are based on absolute counts of circulating alkaline phosphatase positive granulocytes and are therefore not directly comparable to the results which depend on scoring technique.

row cells cultured in a diffusion chamber system [8]. These data appear to support my outlined concept on the meaning of the granulocyte alkaline phosphatase activity.

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Spontaneous Platelet Aggregation in Myeloproliferative Disorders

A Preliminary Study

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Abstract. Spontaneous platelet aggregation has been studied both in normal subjects and in a group of patients affected by chronic myelogenous leukaemia, myelofibrosis, polycythaemia vera and thrombocythaemia. Cold-induced spontaneous aggregation occurs both in normals and in patients. Platelet-rich plasma derived from 13 out of 23 patients, spontaneously aggregated even when kept at 23°C. Spontaneous platelet aggregation was inhibited by aspirin.

Key Words

Aspirin
Myelofibrosis
Myelogenous leukaemia
Platelet aggregation
Polycythaemia vera
Thrombocythaemia

Thrombotic and haemorrhagic complications are well documented in myeloproliferative disorders [7, 9]. Recently NEWMAN *et al* [6] and CARMIGNONE *et al* [2] demonstrated some degree of platelet function abnormality in patients with bleeding diathesis. The mechanisms underlying the thrombotic complications have not been satisfactorily elucidated.

The present study seems to indicate that platelets, in some patients with myeloproliferative diseases, show an abnormal tendency to spontaneous aggregation.

Materials and Methods

Platelet-rich plasma (PRP) was prepared from venous blood collected in plastic tubes taking care to avoid excessive mixing. The blood was anticoagulated with 1:10 of 3.8% sodium citrate and centrifuged at room temperature for 15 min at 600 rpm.

Platelet-poor plasma (PPP) was obtained by centrifuging PRP at 500 rpm for 15 min.

After an equal platelet count, PRP was diluted with PPP to give a platelet count of $400,000/\text{mm}^3$.

Table 1 Spontaneous aggregation (SA) of PRP kept at 23°C for 2 h

	Number of cases	Platelets $\times 10^3$	White blood cells $\times 10^3$	Cases with SA	Latency time, min (mean \pm SD)
Normal	15	200-300	5-8	none	
Chronic myelogenous leukaemia	10	170-600	12-152	6	7 \pm 4
Myelofibrosis	5	140-300	10-15	2	12
Polythaemia vera	7	350-500	10-18	4	7 \pm 2
Thrombocythaemia	1	500	10	1	12

Each test has been carried out at least twice on different occasions

1 ml of PRP, with known platelet number, was distributed into glass test tubes

All the glassware was silicone coated (Siliclad Clay Adams). Platelet aggregation was measured turbidometrically in Mustard's aggregometer [5] attached to a Brush Lomb recorder. One ml sample of PRP was placed in the cuvette holder of the aggregometer which stirred the PRP at 1000 rpm. The tube holder was kept at 37°C. Spontaneous aggregation was tested immediately after storage for 2 h of PRP at 37, 23 (room temperature) and 4°C (refrigerator).

When spontaneous platelet aggregation was detected in the aggregometer, this was confirmed by either gross inspection or microscopy. Platelet counting was performed according to FALUOMO and DI NI [8].

15 normal subjects and 23 patients (table 1) were investigated.

Results

Chilled platelets spontaneously aggregated in normal as well as in patients. Platelets of normal subjects did not aggregate when kept at room temperature or 37°C. On the contrary, PRP derived from patients showed, in 13 out of 23, spontaneous aggregation at 23 and 37°C (table 1). Some examples of spontaneous platelet aggregation curves obtained in normals and patients are given in figure 1.

The supernatant, derived from patients' PRP kept at 23 or 37°C, after spontaneous aggregation was still able to induce platelet aggregation of normal fresh PRP (fig. 2). Platelet aggregation of normal fresh PRP was also induced by adding the supernatant PPP derived from the normal PRP kept at 4°C after spontaneous aggregation.

Aspirin administration in a single dose of 500 mg abolished the spontaneous platelet aggregation at 4, 23 and 37°C in all cases.

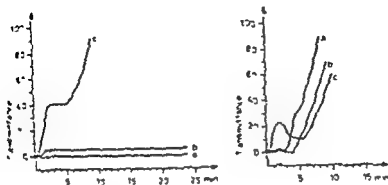


Fig 1 Changes in transmittance during spontaneous platelet aggregation (mean of 3 determinations). On the left, normals; on the right, patients with chronic myelogenous leukaemia. a = PRP kept at 23°C, b = PRP kept at 37°C, c = PRP kept at 4°C.

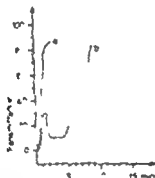


Fig 2 Platelet aggregation curves obtained with 0.9 ml of fresh normal PRP + 0.1 ml of supernatant after aggregation (a) or 0.95 ml of fresh normal PRP + 0.05 ml of supernatant after aggregation (b).

Discussion

ZUCKER and BORRILL [11] and ANDRILL and HAWKEY [1] reported that platelets when incubated at low temperatures, spontaneously aggregate. RASTIN, KATTELON and ALEXANDER [3] showed that cold-induced aggregation resembles ADP-induced aggregation in that the shapes of both aggregation curves are similar. Moreover both aggregations are in-

hibited by the same antiaggregant compounds. Cold induced aggregation was considered not to be mediated by ADP, since this compound was not found in the supernatant of chilled PRP.

Our results confirm that cold induced aggregation is present both in normal and in patients affected by chronic myelogenous leukaemia, myelofibrosis, polycythaemia and thrombocythaemia. In addition we have observed that PRP, in 56% of these patients spontaneously aggregated even when kept at 23 and 37 °C. This aggregation was exactly the same as that normally seen when collagen is added to normal plasma. We observed that supernatant derived from PRP at the end of the aggregation, was able to induce platelet aggregation of normal fresh PRP. The kind of such curve was similar to ADP-induced curve. These phenomena were inhibited by aspirin.

There are no evidences to explain our findings, however, these preliminary results suggest that spontaneous platelet aggregation might underlie the thrombotic complications in patients with myeloproliferative diseases. In this connection VREEKEN and VAN AKEN [10] and MUNDALL *et al* [4] demonstrated a clear relationship between abnormal spontaneous platelet aggregation and transient attacks of vascular ischaemia in 2 patients affected by polycythaemia and thrombocythaemia.

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Haemoglobin O Indonesia ($\alpha 116$ Glutamic Acid \rightarrow Lysine) in an Iranian Family

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Abstract During surveys for abnormal haemoglobins in Iran a number of known and unknown variants of haemoglobin A were found. In one family, a case of Hb O Indonesia $\alpha 116$ Glu \rightarrow Lys was found. The presence of this haemoglobin was not associated with clinical symptoms.

Key Words Haemoglobin O
Haemoglobinopathies
Iran

Haemoglobin O Indonesia ($\alpha 116$ glutamic acid \rightarrow lysine) was first observed by LIE-LOO and SODONO [11], in Indonesia, and its structure was determined by BAGLIONI and LEHMANN [1]. We have recently observed this haemoglobin in an Iranian family during surveys for abnormal haemoglobins on 15,000 patients from University hospitals in which a number of known and unknown variants of haemoglobin A (Hb A) were found [13, 14]. It is not surprising to observe abnormal haemoglobins so frequently among Iranians, since, during centuries, many nations migrated through this country from far east and India to the near east and Europe and, after numerous invasions, groups of people remained and inhabited in some part of the country. One can now trace these ancient populations by studying haemoglobin types of different ethnic groups living in Iran.

Methods

The proband is a 32 year-old man apparently healthy, no other members of his family were available for study. Routine haematological examinations were within normal range according to the standard techniques [18]. No inclusion bodies were found in the red cells. Sick cell preparation was negative. Solubility test was



Fig 1 Starch gel electrophoresis, tris EDTA borate buffer, pH 8.1. Above: Hb A; Hb S; Hb A₂ below: Hb A ~ Hb O

with in the normal range [9] and fetal haemoglobin was less than 1% [16]. Heat denaturation was negative as carried out according to Gomers *et al* [7] and Carazzi and Kay [3].

Results

On electrophoresis on cellulose acetate [6], the variant moves as Hb S. The proportion of the abnormal fraction, was 20% as estimated by elution of haemoglobin bands from cellulose acetate after electrophoresis [12]. In starch gel electrophoresis with tris EDTA borate buffer, pH 8.1, the variant moves slightly faster than Hb A₂ (fig 1), the variant separates from Hb A after electrophoresis in agar gel at pH 6.2 [15], with the same mobility of Hb C.

The abnormal haemoglobin was purified by column chromatography of the haemolysates on DEAE-cellulose (Whatman DE-52), using tris-HCl buffer, pH 8 and 0.05 M [8]. The variant was separated from Hb A₂ and obtained in a pure form. Globin was prepared by the treatment of the purified Hb D with 2% concentrated HCl in acetone at -20°C, washed with cold acetone and freeze-dried.

To identify the abnormal polypeptide chain, electrophoresis of the globin was carried out in starch gel containing 6 M urea and 0.05 M 2-mercaptoethanol [4] which revealed an additional slow moving band in

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Fig. 1. Starch gel electrophoresis, tris EDTA borate buffer pH 8.1. Above: Hb S, Hb A₂, below: Hb A + Hb O.

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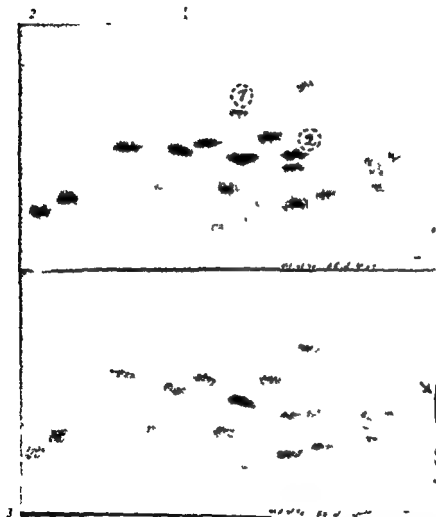


Fig 2 Electrophoresis of the globin in starch gel containing 6 M urea and 2 mercaptoethanol. Note the additional slow moving α -chain.

Fig 3 Fingerprints of the S-aminoethylated α -chain from Hb O (above) and Hb A (below). Arrow indicates the usual place of the peptide α TP 12b. 1 and 2 are 2 new peptides which both stain for histidine.

Table 1

Amino acid	Peptide 1	Peptide 2
Lysine	1	2
Histidine	1	1
Aspartic acid	-	1
Threonine	1	1
Serine	-	1
Proline	1	1
Alanine	3	2
Valine	1	1
Leucine	4	1
Methionine		1

(fig 2) suggesting that the abnormality rested in the α -chain. This was confirmed by the electrophoresis of the haemolysates on cellulose acetate [17] as well as treatment of the haemolysates with *p*-hydroxymercuribenzoate (PHMB) [2], followed by starch gel electrophoresis, pH 8.6.

A Polypeptide chain from Hb O was prepared by CMC column chromatography with phosphate buffer, pH 6.7, containing 6 M urea and 0.05 M 2-mercaptoethanol followed by aminoethylation [5]. Trypsin digestion of 5 aminoethylated normal and abnormal α -chain and fingerprinting was carried out according to Cline *et al.* [5], except that isoamyl pyridine H_2O (10:30:35) was used for chromatography. Figure 3 shows the fingerprints of the aminoethylated peptides from Hb O as well as normal α -chain. The peptide α TP 12b is missing from its usual place (arrow) and 2 new peptides, 1 and 2, are present in the fingerprint of Hb O, both of these peptides stain for histidine.

The abnormal peptides were each eluted from 6 preparative fingerprints with 6 N HCl and hydrolysed for 20 h at 105 °C in methoxyacetic acid. HCl was removed by rotary evaporation and their amino acids were determined on an automatic amino acid analyser (Hitachi KLA-5). Table 1 shows the amino acid contents of the 2 extra peptides 1 and 2.

The results of the amino acid analysis of the 2 extrapeptides indicate that in this variant residue 11b of the α -chain glutamic acid has been replaced by lysine and after trypsin digestion this lysine has been cleaved by trypsin resulting the peptide α TP 12b is split to 2 new peptides which are present in the fingerprints of Hb O 1 and 2 in Figure 3.

The amino acid composition of the extra peptide 1 is the same with the first part of the peptide α TP 12b, namely residue 104–116, except glutamic acid which is replaced by a lysine, and the amino acid composition of the extrapeptide 2 is similar to residue 117–127 of the α -chain.

The substitution of ϵ lysine for a glutamic acid has rendered Hb O to have 2 additional positive charges from Hb A, as detected from its electrophoretic mobility at alkaline pH.

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Übergang einer Polycythaemia vera in eine akute Monozytenleukämie

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Abstract In a 68 year old male patient a polycythemia vera underwent a transformation into an acute leukemia. The dominant cells could be identified as monocytes by cytochemical staining. This observation can be used as sign of the myelogenic origin of the monocytes.

Key Words
Cytochemistry
Leukemia following
polycythemia
Monocyte origin
Monocytic leukemia
³²P therapy
Polycythemia

Übergänge der Polycythaemia vera (PV) in eine akute Leukose werden nach Radio-Phosphor-Therapie in 10-14% der Fälle beobachtet [7, 21, 22]. Es ist umstritten, ob es sich hierbei um einen direkten Effekt der ionisierenden Strahlen handelt [12, 13], oder ob durch die bessere Lebenserwartung die Chance wächst, das der Krankheit innewohnende Risiko der Transformation in die Wucherung unreifer Zellen zu erleben [14, 15].

Die Leukosen werden in der Regel als «akute myeloische Leukämien» [2, 9], zum Teil auch als «Paramyeloblastenleukämien» [16, 19] bezeichnet. Zu unserer Überraschung konnten wir in einem Fall die sich aus einer PV entwickelnde akute Leukose als Monozytenleukämie klassifizieren. Zytochemische Untersuchungen über entsprechende Krankheitsverläufe sind uns nicht bekannt. Lediglich wurde im Einzelfall bei einer chronischen Lymphadenose der Übergang in eine Monozytenleukämie beschrieben [4]. So mochten wir über diesen Krankheitsverlauf berichten, zumal er einen weiteren Beitrag zur Diskussion um die Herkunft der Monozyten darstellt.

Krankengeschichte

G.B., geb. 1904, Nr. 12443 Familienanamnese unauffällig 1929 Appendektomie, 1941 Tonsillektomie, Cholezystitis. 1946 wurde der Verdacht auf eine Polycythaemia geäußert. Im November 1957 erstmals Untersuchung in der Medizinischen Universitätsklinik Göttingen. Erwaß reduzierter Allgemeinzustand, ausgeprägte Ruhrözyanose, vermehrte Blutfülle der Schleimhäute. Leichte Vergrößerung der Leber (+2 Ql). RR 200/120 mm Hg. Hb 22,5 g% Erythrozyten 792 Millionen/mm³, Hämatokrit 80%, Thrombozyten 620.000/mm³, Leukozyten 10.900/mm³ (Stab R, Seg 58, Fo 3, Bazo 0, Lympho 24 Mono 7%).

Im September 1956 Bestätigung der Diagnose durch histologische Untersuchung einer Beckenlammbiopsie (Abb. II) sowie durch Bestimmung des Erythrozyten- und Plasmasolumens. Vom November 1957 bis März 1972 Behandlung mit wiederholten Aderlässen und insgesamt 40 mCi ³²P, verteilt auf 9 Einzeldosen. Nach der Behandlung bis 1971 immer wieder vorübergehende Besserung der durch die PV verursachten Beschwerden. Seit 1967 rezidivierende Thrombophlebitiden. 1969 Lungenembolie. 1969 Nachweis einer sekundären Hyperurikämie mit Uratsteinen. Seit 1970 leichte Retention harnpflichtiger Substanzen. 1970 Diagnose einer Au-Ag positiven Hepatitis durch Leberbiopsie.

Im März 1972 erneute Aufnahme wegen Atemnot, Kopfschmerzen, Nachblasen der Leistungsfähigkeit. Klinisch trotz Digitalisierung Zeichen der Herzinsuffizienz, außerdem nach wie vor Anzeichen eines Vollbildes der Ph. Ruhrözyanose, inguinierte Konjunktiven, Milztumor (+2 Ql). Hb 20,1 g%, Erythrozyten 964 Millionen/mm³, Hämatokrit 70%, Thrombozyten 365.000/mm³, Leukozyten 10.400/mm³ mit Linkverschiebung (Stab 5).

In der Beckenlammbiopsie war die Granulozytopenie gegenüber 1956 in der Reifungsphase stärker Linkverschieben. Im Übrigen wie früher enorm gesteigerte Zellzahl, die durch Vermehrung vorwiegend der erythropoetischen Zellen, aber auch der zum Teil in Neutrophilen liegenden Megakaryozyten (Abb. 2) Erneute ³²P-Therapie (4 mCi). Bei gleichbleibendem Befinden im Mai 1972 Abfall des Hb auf 14,4 g%, der Leukozyten auf 2.000/mm³, im Differenzialausstrich erstmals 16% unreife Zellen.

Juli 1972 Hohes Fieber, allgemeine Schläfiche Zunehmende Hepatosplenomegalie. Hb Abfall auf 9, später 7 g%. Anstieg der Leukozyten auf 20.000 (10.000/mm³) (4) bis zum terminalen 94% Blasten im Knochenmark nahezu ausschließlich grobe unreife Zellen, deren Kern ein lockeres Chromatingesetz und mehrere grobe Nukleolen enthält und deren Zytoplasma sich nur ungleichmäßig anfärbt. Bei der zytochemischen Analyse erhalten diese Zellen deutlich eine extramitotische Granula, dabei nur gelegentlich eine schwach positive Reaktion auf Peroxidase. PAS-Reaktion negativ (Abb. 3) (morphol. AS-Färbung Färbung nach Leitz 101, Peroxidase nach Graham-Knoll und PAS-Reaktion JJ). Trotz hochdosierter zytostatischer Behandlung Verschlechterung des Allgemeinzustandes mit terminalem Blasenversagen auf Werte von 20000 mm³. Am 24. September 1972 Exitus an Kreislaufstillstand. Die eine Stunde post mortem durchgeführte Beckenlammbiopsie zeigte eine diffuse Durchsetzung des Knochenmarks mit unreifen reifen Zellen, deren Kern zum Teil leicht gebuchtet erscheint, vorwiegend blühende Zellen (Abb. 4).

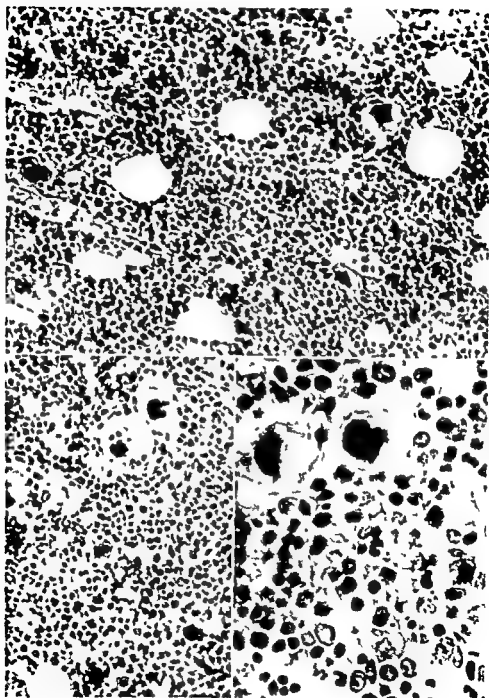


Abb 1 Beckenkammibiopsie 1966 Gesteigerte Zelldichte durch Hyperplasie der Erythro-, Granulo- und Thrombopoese Weite S nusoid HE, $\times 270$

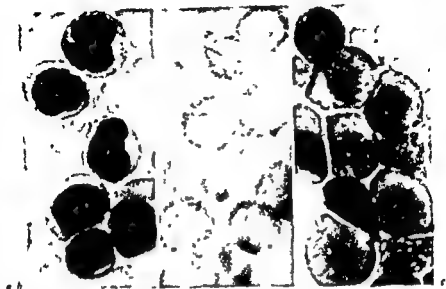


Abb. 1 Blutausstrich im terminalen Blasterstich: a Pappenheim-Färbung. In den zum Teil gebildeten Kernen grosse Nukleolen. b Positive Esterase-reaktion, c Peroxydase-reaktion nur vereinzelt schwach positiv. Zum Vergleich stark positive Reaktion bei einem Segmentkern (gelblich). 1000

Sektionsbefund Nr. 601/72: Rote Metaplasie des Femurmarkmarkes. Hepatomegalie (Leber 270 g, Milz 140 g), Lymphknotenschwellungen paraaortal bis hilargross. Zentral ulzerierte flache gummiartige Tumorknoten in Sigma und Rektum. Mikroskopisch im Femurmarkmark Milz, Lymphknoten sind die Durchsetzung mit unreifen Zellen wie in Abbildung 4. Gleichartige Zellinfiltration in Sigma und Rektum, periaortal bis weit in der Leber, ferner herdförmig in den Nebenhieren und den Nieren.

Nebenbefunde: Intertracheale Erythrozytenperipherie, Nephrosen mit Glomerulonephritis, Basalmembranverdickungen der Glomerulumschlingen und Perikarditis der Leber. Cholestase, cholestische Cholezystitis. Periportale Lebernekrose in der Leber mit Gallengangsverengungen und schütterem Rundzellinfiltrat.

Abb. 2 Reizkollisionskörper März 1972: Weisse Zellen, b geringe Zellzahl durch Hyperplasie vorwiegend der Erythro- und Thrombozytose mit unvollständigen Erythrozyten. c Riesenzellen treten gegen bei den erythropoetischen Zellen massenhaft und sind innerhalb der Reizkörper nachfolgend. Abb. 1 wegen der starken Zellvermehrung bei Myelofibrose (vgl. Gaudner 1972b, S. 43).

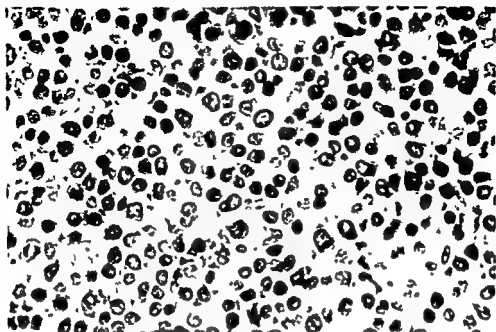


Abb 4 Unmittelbar postmortal entnommene Beckenkammibiopsie. Dichte Durchsetzung des Knochenmarks mit unreifen Zellen. Goldner $\times 490$

Diskussion

Nach 16-jährigem Verlauf ging bei diesem Patienten die PV in eine akute Leukämie über. Bei der zytochemischen Differenzierung der Blasten sprach der Nachweis der unspezifischen Esterase unter Verwendung von Naphthol-AS-Azetat als Substrat bei nur sehr schwach positiver Peroxydase- und negativer PAS-Reaktion dafür, dass es sich um eine Monozytenleukose handelte [1-3, 11, 18]. Bei der Pappenheim-Färbung wie auch bei der histologischen Untersuchung fielen nur gelegentlich leichte Kerneinschnürlungen auf. Im übrigen entsprach der postmortale Befund dem anderer terminaler Leukosen bei PV. Weitere zytochemische Untersuchungen, Hautfärbungen oder die Lysozymbestimmung im Serum und Harn [17, 18] waren wegen des raschen Verlaufes nicht mehr möglich.

Im Schrifttum ist man kaum auf die Frage eingegangen, um welche Art von Blasten es sich im Terminalstadium einer PV handelt. Es wird von Paramyeloblasten [16], auch von monozytoiden Blasten [14, 19] ge-

sprochen ohne dass dieser Eindruck durch zytochemische Untersuchungen belegt werden konnte. Bei unserer Beobachtung handelte es sich um Zellen, die zytochemisch als Monozyten zu differenzieren waren. Man muss also annehmen, dass nach der zur Ausreifung befähigten Proliferation aller blutbildenden Zellen eine zweite Krankheitsphase folgte, in der allein Monozyten gebildet wurden bzw. die zur Proliferation stimulierten Stammzellen sich allein zu Monozyten entwickelten.

Bei der Diskussion, ob die Monozyten den myeloiden Zellen im engeren Sinne zuzurechnen sind [Literatur S. 8, 17] oder ob sie dem retikuloendothelialen System (RES) entstammen [Literatur S. 16] ist diese Beobachtung als ein weiterer Hinweis auf die myelogene Entstehung der Monozyten zu werten. Es ist nahelegend, dass die zuvor gesteigerte Proliferation myeloider Zellen auch terminal im undifferenzierten Wachstum myeloider Zellen endet. Das gilt besonders, da bei der PV das gleichzeitige oder terminale Vorkommen vom RES ausgehender Tumoren oder Systemerkrankungen nicht beschrieben wurde [2, 5, 19, 20].

Die Phase der Umwandlung der PV in eine akute Leukose wird nur ausnahmsweise bei der Knochenmarkuntersuchung erfasst. Auch in diesem Fall wurde das Mark nur vor oder nach der Krankheitstransformation untersucht. 5 Monate vor dem Tode, als die Erythropoese noch maximal stimuliert war, fiel im Knochenmark eine stärkere Linksverschiebung der granulopoetischen Reifungsreihe auf, ohne dass im Blutausstrich unreife Zellen nachweisbar waren (vgl. Abb. 2). So ist es möglich, dass die Blastenwucherung im hyperplastischen Mark begann. Da jedoch der massiven Blastenauswucherung ein Stadium der Zytopenie vorausging, ist als zweite Möglichkeit zu diskutieren, dass die Proliferation der Blasten in einem nach der letzten Radiophosphortherapie phthisisch umgewandelten Mark begann, wie es früher zufällig bei einer histologischen Untersuchung erfassten konnten [6]. Schon wegen des engen zeitlichen Zusammenhanges ist es in diesem Fall wahrscheinlich, dass die Krankheitsumwandlung durch die unmittelbar vorausgegangene Radiophosphortherapie begünstigt wurde.

Zusammenfassung

Bei einem 65-jährigen Patienten entwickelte sich aus einer Polycythaemia vera nach 14 Jahren Verlauf eine akute Leukämie. Die Leukämie konnte eindeutig als Monozytenleukämie differenziert werden. Diese Beobachtung ist als weiterer Hinweis auf die Entstehung der Monozyten aus vorliegenden Zellen anzusehen.

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Ropalocytosis in a Patient with Acute Lymphoblastic Leukemia

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Abstract A patient with acute lymphoblastic leukemia whose red blood cells showed the abnormality ropalocytosis is described. This abnormality is characterized by bizarre pseudopodia like processes detectable in the mature red blood cells and reticulocytes. The presence of ropalocytosis suggests pathology of the hematopoietic system.

Key Words

Erythrocyte morphology
Leukemia
Lymphoblastic leukemia
Ropalocytosis

Alterations of the red blood cell morphology in leukemia, such as anisocytosis, macro- and microcytosis, poikylo- and spherocytosis are well known. Recently, an unusual abnormality of the red blood cells in hairy cell leukemia was reported by GHADIALY and SKINNER [5] and named by the authors ropalocytosis [4]. The authors define this abnormality as consisting of numerous branched and unbranched cell processes protruding from the cell membrane. They can be observed in ultrathin sections examined with the electron microscope.

We had the opportunity to examine a patient with acute lymphoblastic leukemia whose red blood cells showed alterations compatible with those described as ropalocytosis.

Case Report

A 67 year-old German born male was admitted to the department on September 7, 1972 because of a painful ulcer in the inner part of the upper lip. The lesion appeared 1 month prior to admission, healed after a few days without any treatment and reappeared after 2 weeks.

Past history revealed a partial gastrectomy performed 20 years previously because of duodenal ulcer and mild diabetes mellitus kept under control by diet only.

On physical examination, the patient was found to be in poor general condition. Blood pressure was 140/70 mm Hg. On the left side of the upper lip an ulcer could be seen 3 cm in diameter, having regular margins, a white bottom and no hemorrhages. Except for a few palpable lymph nodes in the left submandibular region, 1 cm in diameter, not tender and soft, there were no additional pathological findings.

Laboratory examinations showed hemoglobin 8.1 g%, HtK 25%, reticulocytes 2.2%, total white blood cell count 22,000/mm³, with the following differential count: polymorphonuclears 4%, basophils 2%, monocytes 1%, lymphocytes 80%, and blasts 13%. Platelet count 19,000/mm³. The blood smear revealed marked anisocytosis, macrocytosis and a few spherocytes.

Bone marrow aspiration biopsy revealed a slightly hypoplastic bone marrow, but nuclearity of the cells of the red blood series, marked hypoplasia of the granulocytic series and infiltration by the same lymphocytes and blasts seen in the peripheral blood smear. Serum iron was 163 µg% and serum iron binding capacity 120 µg%, total bilirubin 1.0 mg%, unconjugated 0.7 mg%. Direct and indirect Coombs' tests were negative. The plasma hemoglobin concentration was 2.5 g%. Urea, glucose, creatinine, uric acid, cholesterol, total proteins, albumin, globulin, protein pattern by paper and immunoelectrophoresis, fibrinogen, prothrombin, SGOT and SGPT, were normal. The 24 hour sterco-bilirubin excretion was 132 mg%.

The diagnosis of acute lymphoblastic leukemia was considered. Treatment with prednisone (40 mg/day) and vincristine (2 mg/m²) was started. Two days following the biopsy the patient started to bleed profusely from the operative wound, as well as from the nose. A survey of the coagulation mechanism revealed a reduced euglobulin lysis time of 60 min (normal value 120 min), the presence of fibrinogen degradation products (40 µg/ml), PTT 37 sec (normal 35-45 sec) and fibrinogen 247 mg% (normal 400-600 mg%). Treatment with 16 g/day of amniar (epiuron aminocaproic acid) in addition to repeated blood transfusions stopped the bleeding. The patient's condition improved, but a few days later his temperature rose and pneumonia appeared in the buccal mucosa. Administration of amphotericin B and mycostin did not result any improvement and death occurred 27 days after the admission. Post mortem examination was denied by the family.

Electron microscopy. Buffy coat was obtained from 10 ml venous blood with 4000 in a heparinized syringe fixed in 1% cold glutaraldehyde pH 7.4 for 2 h and post fixed in 1% osmium tetroxide. The cells were dehydrated in graded alcohols and embedded in epoxy E12. Thin (400 Å) Al sections were cut with an LKB ultratome III and examined with a Philips EM 10 electron microscope.

Some of the red blood cells and reticulocytes revealed a bizarre and compressed shape (Fig. 1). They showed pseudopodia like processes with clubbed ends usually protruding from a limited portion of the cell membrane although in a few instances complete irregularity of the cell membrane was noted (Fig. 2). In some of the cells (Fig. 3) a membrane ring was demonstrated whereas other cells showed numerous and large vesicles, a few containing cytoplasmic debris. They were char-

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The Effects of Irradiation on the Haematopoietic Tissues of Anaesthetized Mice

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Abstract The effects of irradiation on the haematopoietic tissues of anaesthetized mice have been measured using the balance between damage and recovery during the 10-day period following the administration of various doses of whole body λ irradiation. The cellularity of the femoral bone marrow, the weight of the spleen and the number of (endogenous) spleen colonies are elevated in animals anaesthetized using Avertin during exposure to doses of whole body λ irradiation in the range between 400 and 850 rad with a dose reduction factor of 1.2-1.4.

Key Words

Anaesthesia
Bone marrow
Irradiation
Lymphoid tissues
Mouse haematopoiesis
Spleen colonies

Studies of the radiosensitivity of haematopoietic stem cells and of haematopoietic recovery in partially irradiated animals which can provide extremely useful information, often necessitate the irradiation of immobilized, anaesthetized animals [2, 3, 6]. It is, therefore, important to recognise the possibility that anaesthetics which have not been subjected to systematic evaluation may distort the effects of irradiation [1]. A weak protective effect has been observed after the administration of ethyl alcohol but this could not be attributed to its anaesthetic properties and neither nembutal nor ethyl carbamate modified the survival patterns of irradiated mice [7].

VAN BEKKUM [13] has, however, reported an increase in the LD 100/30d minimum in mice which were immobilized while they were exposed to whole body λ -irradiation. Subsequent investigations have revealed that both physical restraint and Nembutal anaesthesia enhance the survival of bone marrow CFU, while several other anaesthetics increase the LD 50/30d without influencing the gastro intestinal syndrome [4, 5].

The effects of irradiation on the haematopoietic tissues of mice anaesthetized using Avertin have been studied in the present investigation, which forms part of a series of experiments designed to analyse the effects of whole body X-irradiation on the haematopoietic and lymphoid tissues of the mouse.

Materials and Methods

Animals and housing. Specific pathogen free female albino mice of the C57 strain (supplied by Scientific Products Farm, Ash, Kent) weighing between 20 and 25 g were used throughout this investigation. The mice were housed 5 to a cage in a laminar flow cabinet and given boiled water and mouse chow (41B and 44) *ad libitum*.

Irradiation and anaesthetic. Mice were irradiated in the radially disposed compartments of a circular cage (300 kVp, 5 mA, 0.5 mm copper plus 5 mm aluminium) at a dose rate 64 rad/min.

Avertin was an amylose hydrate (indroethanol, Wierthrop Laboratories, New York) was made up in warmed normal saline and 0.01 ml/g body weight (300 mg of Avertin/kg body weight) was injected intraperitoneally to anaesthetize the mice for the duration of the irradiation.

Experimental design. Eight groups of not less than 10 mice received doses of X irradiation in the range between 200 and 840 rad. The LD_{50/30d} for C57 mice is 700 rad. Five groups of not less than 7 anaesthetized mice were also irradiated. After 10 days, the irradiated mice were killed by exposure to ether vapour. The spleen, thymus, inguinal lymph nodes and brachial lymph nodes were dissected and weighed. The cellularity of the femoral bone marrow was measured by syringing the contents of the medullary cavity of the femoral diaphysis into 20 ml of fixation and determining the cell content of the resultant suspension using a Coulter Counter. After the spleen had been fixed in aqueous Bouin's fluid it was transferred to 70% alcohol and macroscopic surface colonies were subsequently counted.

Results

The average numbers of nucleated cells in the femoral diaphysis and the weights of the spleen, thymus and pooled lymph nodes have been plotted for the control and anaesthetized groups of irradiated mice (Fig. 1-4). In both groups all of these parameters decrease as the dose of X irradiation is increased. The cellularity of the femoral bone marrow and the weight of the spleen are, however, elevated in the groups which were irradiated and anaesthetized ($P < 0.05$ for both at 600 rad). The dose reduction factor (DRF) in the range between 400 and 840 rad is about 1.2-1.4 for these tissues. The number of endogenous spleen

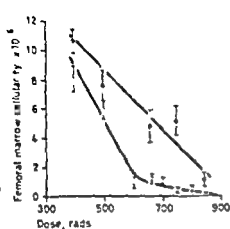
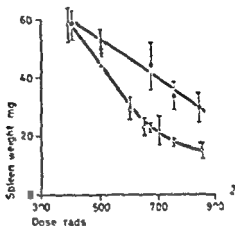


Fig 1 The cellularity of the bone marrow 10 days after the exposure of mice anaesthetized using Avertin (●) and of unanaesthetized controls (○) to various doses of whole body X irradiation

Fig 2 The weight of the spleen 10 days after the exposure of mice anaesthetized using Avertin (●) and of unanaesthetized controls (○) to various doses of whole body X irradiation



colonies is similarly elevated with a similar DRF (fig 5). Most of the spleens in the anaesthetized mice which received 500 rad of whole-body X-irradiation exhibited confluent areas in which individual colonies could not be discerned, whereas in the control group the surface colonies were not confluent after this dose of X-irradiation. The weight of the thymus was elevated relative to the weight of the thymus in controls only in anaesthetized animals exposed to the highest doses of X-irradiation administered, and the weight of the lymph nodes was not affected by Avertin anaesthesia ($p > 0.45$ and 0.9 at 670 rad).

Discussion

Avertin anaesthesia modified the effects of whole-body X-irradiation on the haematopoietic and lymphoid tissues of the mouse in a manner which is comparable to that recently reported for several other anaesthetics [4, 5]. The CFU content of the femoral bone marrow is elevated in animals anaesthetized with Nembutal before being irradiated and it has been suggested that the beneficial effect of the anaesthetic may be due to hypoxia [4, 5]. In the present investigation Avertin anaesthesia has been

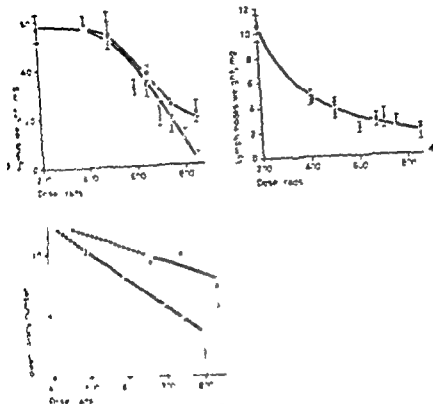


Fig. 1. The weight of the spleen 10 days after the exposure of mice anaesthetized using Avertin (●) and of non-anaesthetized controls (○) to various doses of whole-body γ -irradiation.

Fig. 2. The weight of the spleen 10 days after the exposure of mice anaesthetized using Avertin (●) and of non-anaesthetized controls (○) to various doses of whole-body γ -irradiation.

Fig. 3. The number of mitotic colonies observed on the 10th day after the exposure of mice anaesthetized using Avertin (●) and of non-anaesthetized controls (○) to various doses of whole-body γ -irradiation.

shown to exert beneficial effects on the cell counts of the bone marrow and the mass of the spleen as well as on the number of colonies formed in the spleen. The studies which have been shown to result in a significant improvement after the administration of whole-body γ -irradiation are those which have been reported in the literature. The results of the

lymph nodes [10-12], benefits only slightly from Avertin anaesthesia during the period of irradiation. The mass of the lymph nodes is not elevated appreciably in anaesthetized irradiated animals in contrast to the cellularity of the bone marrow and the mass of the spleen. This contrast may reflect the absence of regeneration in the lymph nodes after the administration of all doses above 415 ± 40 rad during the 10 day period following whole body X irradiation [8], so that regeneration would not be expected to occur in the lymph nodes unless the dose of X-irradiation administered was rendered comparable in its effects to a dose of less than 400 rad.

It is thus essential to take into account the actions of anaesthetics in order to be able to utilize the effects of irradiation on the haematopoietic tissues in assay systems which depend upon the use of anaesthetized irradiated mice. It would be useful to compare the effects of Avertin and of similar anaesthetics on the haematopoietic tissues with their effects on the immune response.

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Morphologie de l'érythropoïèse chez *Lacerta muralis* (Laurenti)

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Abstract Although the erythrocytes of lizard are clearly different from those of mammals (persistence of the nucleus and of a residue of cytoplasmic basophilic substance), there is an analogy in their production starting with a large pro-erythroblast, 4 successive mitoses reduce the nuclear size to $1/2$, $1/4$, $1/8$ and $1/16$, allowing differentiation of 5 cell types. The passage from the marrow into the blood, however, is less selective than in mammals: in the blood of lizard one finds numerous cells that have not undergone all the mitoses and transform directly into larger erythrocytes with larger nuclei, than the majority of erythrocytes.

Key Words
Erythroblaste
Karyokinetique
Lizard erythropoiesis

Les différents stades de l'érythropoïèse sont classiquement définis chez les mammifères au moyen de critères morphologiques et tinctoriaux. Quatre stades sont décrits: proérythroblaste à gros noyau nucléolé et à cytoplasme basophile, érythroblaste polychromatophile et érythroblaste acidophile. Chez les autres vertébrés, les stades sont superposables ainsi qu'il ressort des études de YOKOYAMA [15], d'ANDRÉW, d'ORIO [6] chez les poissons, de DE VILLIERS-PINNAIR chez les reptiles, et de LUCAS et JAMROZ [10] chez les oiseaux.

D'intéressantes précisions ont été apportées chez l'homme par l'étude des dimensions nucléaires ou caryométrie. LEBETSEDER [9], WEICKER [13] puis CAZAL [1], appliquant à l'érythropoïèse la méthode générale proposée par JAKONI [7], constatent que la maturation se fait en 5 stades et non en 4, séparés par des mitoses qui divisent chaque fois par deux le volume nucléaire sans que celui-ci se reconstitue (mitoses hétéroplastiques). Les volumes nucléaires de ces 5 stades sont dans les proportions 1, $1/2$, $1/4$, $1/8$ et $1/16$. La cellule du premier stade paraît subir une

mucose hémoplastique, une des cellules filles accroissant son noyau pour reconstituer la cellule-mère, l'autre cellule-fille conservant un volume nucléaire réduit de moitié. Le cytoplasme des stades caryoméiétiques 2 et 3 est basophile, celui du stade 4 est polychromatophile et celui du stade 5 est acidophile. Si l'on conserve donc la terminologie classique, il faut distinguer deux érythroblastes basophiles et non plus un seul.

La présente communication résume les constatations analogues faites chez le lézard et précise certaines particularités.

Matériel et Méthodes

L'étude a porté sur de jeunes lézards des murailles, *Lacerta muralis* (Laurer), mesurant 5 cm de la tête au cloaque. Les individus de taille supérieure sont éliminés car le canal d'apophyse de leurs os longs contient une trop forte proportion de moelle jaune.

Le sang est prélevé à la queue de l'animal, soit directement sur la lame, soit à l'aide d'une pipette capillaire dont la partie interne est recouverte d'une couche d'heparine desséchée.

Pour obtenir la moelle la d'apophyse des os longs est séparée des épiphyses et, à l'aide d'une seringue adaptée à une extrémité, la moelle d'apophyse est relouée dans une pipette sur une lame.

Les frottis de sang et de moelle aussi réguliers que possible sont colorés par la méthode panoptique de May-Griernald-Giemsa. La substance granulo-filaire est toujours en évidence par la coloration vitale au bleu de créosyl.

Les préparations sont étudiées avec soin réalisées sur microphotographies à grandissement 1000 fois. Les surfaces sont mesurées au planimètre. Les rapports surface/volume correspondent à des rapports de surface.

$$\frac{\text{surface mesurée}}{\text{surface vraie de la cellule}}$$

La cellule même peut être étudiée à partir de mesures linéaires au planimètre. Ces deux procédés ont été utilisés sur un grand nombre de cellules érythroblastiques prises au hasard sur les microphotographies avec mesurées et réparties en classes d'abaissement.

La forme du noyau n'est pas liée à son cercle de diamètre moyen ou la mesure au planimètre de son diamètre moyen. Les noyaux ovales dont les diamètres moyens diffèrent entre eux de plus de 10% ont cependant une même surface. Il est donc intéressant de se rendre compte de cette donnée sur le diamètre moyen les mesures de surface sont plus précises que celles obtenues par la mesure des diamètres.

Les érythrocytes sont étudiés d'une étude morphologique faite à partir d'un échantillon de 1000.

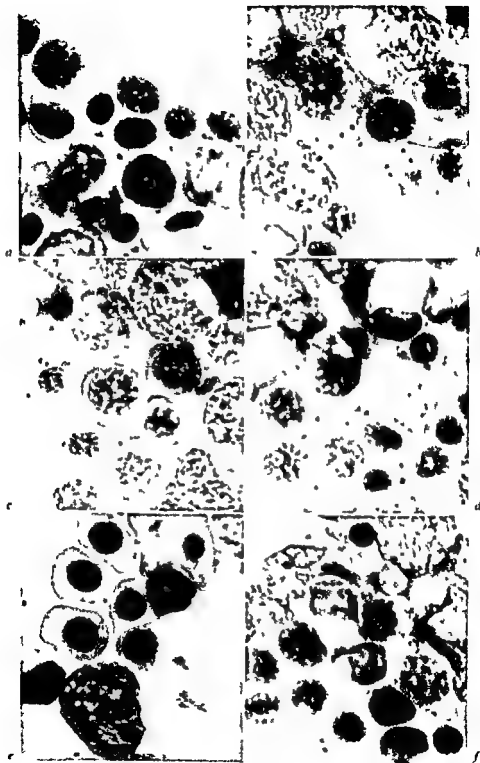


Fig. 1

Résultats

La lignée érythrocytaire dans la moelle

Les frottis de moelle contiennent des érythroblastes et des érythrocytes (fig 1). La distinction entre eux est facile en raison du noyau allongé et de la grande taille cytoplasmique des érythrocytes. Pour les courbes earyométriques, seuls les érythroblastes ont été pris en considération.

Les deux courbes de fréquence obtenues par l'étude statistique de 200 cellules peuvent être interprétées de façon identique et permettent de différencier les stades évolutifs qui se marquent par des pics de la courbe mieux délimités sur la courbe des surfaces (fig 2). Cinq pics sont identifiables, correspondant à 5 stades érythroblastiques, tout comme chez l'homme. Un simple calcul montre que le volume nucléaire de chacune de ces classes correspond à la moitié de celui de la classe suivante.

Ces 5 stades seront désignés ici par les noms de proérythroblaste, érythroblastes I, II, III, et proérythrocyte. Leur identification métrique permet d'en décrire les caractères morphologiques (fig 1). Des mitoses sont repérables dans les 4 premiers stades, mais non dans le 5e, d'où un nom impliquant une transformation sans mitose en érythrocyte.

Le proérythroblaste est le plus volumineux (diamètre 12-14 μ m), le noyau circulaire ou ovalaire, central, a un diamètre moyen de 10,7 μ m, son réseau chromatinien est fin et régulier, et il contient 2-5 nucléoles de forme peu nette, le cytoplasme homogène, franchement basophile avec un liseré périmukaire plus clair, est peu abondant (rapport N/P de 0,9 environ).

Fig. 1. La lignée érythroblastique dans le frottis de moelle. 670 x. Un proérythroblaste au centre, un grand érythroblaste basophile en haut et à gauche, un érythroblaste plus érythrocytaire en haut et à droite, un érythrocyte en bas et à droite et à gauche et de gauche à droite une forme proérythroblastique, II grand érythroblaste basophile au centre, 2 grands érythroblastes basophiles et un érythrocyte plus érythrocytaire et 3 petits érythroblastes basophiles en bas et à gauche, 2 érythrocytes plus érythrocytaires au centre, deux et 4 érythroblastes plus érythrocytaires et un érythrocyte et un érythrocyte en proérythrocyte en une de ces et un érythrocyte légèrement ovalaire cytoplasmique en une de droite, un érythrocyte et un érythrocyte en bas, une forme érythroblastique plus érythrocytaire en bas et à gauche, 2 érythrocytes plus érythrocytaires en bas au centre et à droite.

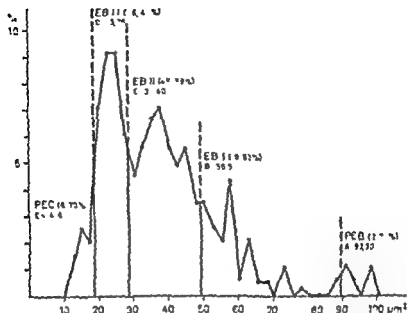


Fig 2 Fréquences érythroblastiques médullaires selon les surfaces nucléaires Les 5 stades érythroblastiques sont caractérisés par leur surface moyenne respective A B C D E F PIB = Proérythroblaste, EBI = grand érythroblaste basophile EBI = petit érythroblaste basophile, EBI = érythroblaste polychromatophile PEC = érythroblaste acidophile ou proérythrocyte

L'érythroblaste I est plus petit, le diamètre nucléaire moyen est de 8,5 μm , les nucléoles ont disparu et le réseau chromatinien a tendance à se condenser en certains points, le cytoplasme est franchement basophile le rapport N/P est de 0,7

L'érythroblaste II a un noyau plus réduit (diamètre moyen 6,8 μm) a chromatine condensée en petites mottes bien distinctes, le cytoplasme est basophile le rapport N/P est de 0,55

L'érythroblaste III a un cytoplasme polychromatophile, ce qui signe la diminution de la substance basophile cytoplasmique (ARN) et l'apparition de l'hémoglobine, le noyau a un diamètre moyen de 5,3 μm , la chromatine est condensée en mottes régulières, le rapport N/P est de 0,5

Le proérythrocyte est la cellule la plus petite de la lignée, plus petite même que les érythrocytes dont la condensation nucléaire est largement compensée par l'accroissement cytoplasmique, son diamètre varie entre 6 et 8 μm son diamètre nucléaire moyen est de 4,5 μm , la chromatine est condensée en quelques gros blocs réguliers, le cytoplasme est acido-ophile le rapport N/P dépasse encore 0,4

La fréquence médullaire des 5 stades n'est pas superposable à ce que l'on constate chez l'homme ou le nombre double lorsque le volume nucléaire est divisé par deux. Si le même rapport est retrouvé pour les érythroblastes I et II (20 et 40^e), l'érythroblaste III est moins abondant que l'érythroblaste II (26^e seulement) et le proérythrocyte est rare (67^e). Ces chiffres indiquent soit qu'une partie de la production médullaire suit vers le sang soit que la durée des différents stades diminue avec leur degré de maturation, les derniers étant les plus courts.

La lignée érythrocytaire dans le sang

L'érythrocyte est une grande cellule elliptique, de 13 à 15 μ m de long sur 7,5-9 μ m de large. Le cytoplasme est très abondant (rapport N/P 0,16). Le noyau est allongé dans le même sens que la cellule, sa longueur est de $5,9 \pm 0,2 \mu$ m, sa largeur de $2,54 \pm 0,54 \mu$ m. Comme l'ont écrit de nombreux auteurs chez tous les vertébrés possédant des érythrocytes nucléés, la coloration vitale au bleu de créosol brillant met en évidence dans le cytoplasme un matériel basophile granuleux, la conservation du noyau a comme corollaire la conservation d'une partie de l'ARN cytoplasmique. Cette substance basophile se présente comme la substance réticulée filamenteuse des réticulocytes des mammifères.

4^e Les cellules rouges du sang sont des proérythrocytes identiques aux proérythrocytes médullaires et c'est dans le sang que peut être étudiée la maturation du proérythrocyte, c'est-à-dire sa transformation en érythrocyte. Cette maturation se caractérise à la fois par l'accroissement considérable du cytoplasme avec réduction du rapport N/P qui passe de 0,4 à 0,1 et par l'allongement de la cellule et du noyau. L'accroissement cytoplasmique est tel que la cellule grandit. Un stade intermédiaire est souvent observé, cytoplasme abondant et allongé avec noyau encore arrondi ou peu allongé.

A côté de la série proérythrocyte-érythrocyte telle qu'elle vient d'être décrite, on trouve dans le sang des cellules à noyau plus volumineux, dont les caractères morphologiques sont ceux des érythroblastes, ou se rapprochant de ceux des érythrocytes. Les premières sont reconnaissables comme des érythroblastes surtout du stade III (670^e) mais aussi du stade II (4^e) et surtout du stade I (33^e). Les secondes sont de grands érythrocytes (634^e) qui peuvent être considérés en tant que la fin de la série ou, comme il paraît d'après les résultats, comme des érythroblastes III ou exceptionnellement des érythroblastes II. La maturation cytoplasmique se fait sans que le noyau ait été suffisamment réduit.

de volume la dernière mitose (celle de l'érythroblaste III) et parfois l'avant-dernière (celle de l'érythroblaste II) sont sautées. Ce processus normal chez le lézard, est celui de la mégaloblastose humaine arrêt des divisions érythroblastiques et maturation cytoplasmique

Resumé

Ben que les érythrocytes du lézard soient nettement différents de ceux des mammifères (persistance du noyau et d'un résidu de substance basophile cytoplasmique) leur production est superposable à partir d'un proérythroblaste de grande taille quatre mitoses successives réduisent la taille nucléaire à $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$ et $\frac{1}{16}$ ce qui permet de différencier 5 types cellulaires. Cependant le passage de la moelle dans le sang est moins sélectif que chez les mammifères on trouve dans le sang du lézard de nombreuses cellules qui n'ont pas subi toutes les mitoses et qui se transforment directement en érythrocytes plus grands et à noyau plus grand que la majorité des érythrocytes.

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■ TURA and M. BACCARANI (eds.) *Chronic Myeloid Leukaemia* Haematologica Pa-
via 1972

The book gives an excellent account of the most recent insights in the pathogenetic, cytogenetic, kinetic and immunological aspects of the disease. Therapeutic possibilities are thoroughly reviewed and new approaches are proposed. The book can be highly recommended to hematologists and oncologists.

B. SERRCK Basel

R. GROSS und J. VAN DE LOO (Hrsg.) *Leukämie* Springer, Berlin 1972 700 pp.,
219 fig. DM 148.-

Das Buch gibt einen guten und sehr vielseitigen Überblick über den heutigen Stand in der Diagnostik und der Therapie der Leukämien.

Das Stück über die pathologische Anatomie ist sehr vollständig und gibt für den Kliniker wichtige Informationen über Todesursachen, Übergänge der einzelnen Leukämieformen, infektiöse und hämorrhagische Komplikationen. Ebenfalls sehr eindrücklich sind die zytochemischen und elektronenmikroskopischen Darstellungen. Zytogenetika, auch für die Frühdiagnostik, und die Genetik der Leukämien sind in einfacher und übersichtlicher Weise dargestellt. Die Dynamik und die klinischen Aspekte der Leukämien werden in guter Form aufgeführt. Mehrere einschlägige Artikel behandeln die mehr konventionelle antileukämische Therapie, aber auch die Möglichkeit der Zellsynchronisation und der Immunotherapie werden *in extenso* behandelt. Das Stück über den Ersatz von korpuskularen Blutelementen ist adäquat, geht aber nicht sehr eingehend auf die modernsten Methoden des Granulozytentransfers und der Knochenmarkstransplantation ein. Die Behandlung von Leukämiepatienten in protektiver Isolation und ihre prospektive Bedeutung wird diskutiert. Dieses Buch ist meines Wissens zurzeit die umfangreichste deutschsprachige Darstellung des Problems der Leukämie. Es kann für hämatologisch und onkologisch tätige Ärzte und Bibliotheken sehr empfohlen werden.

B. SERRCK Basel

Table 1 Incidence of pyroglobulæmia in our paraproteimæmic patients

	Number of patients	Pyroglobulæmia
IgG Myeloma	151	1
IgA Myeloma	33	3
IgD Myeloma	2	
Bence Jones myeloma	12	
Waldenström's disease with cryoglobulæmia	39	3
Waldenström's disease with cryoglobulæmia	5	3
Total	250	8

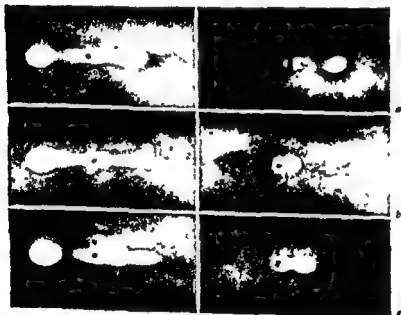


Fig. 1. Photomicrographs of pyroglobulæmia. On the left the smears were from Case M. E. (IgG myeloma) and on the right the smears were from Case E. W. (Waldenström's disease). In Case M. E. IgG myeloma.

Materials and Methods

The presence of pyroglobulins was tested for by warming up to 56–60°C of the serum taken from our paraproteinemic patients. Zone electrophoresis of serum was performed in agar gel using Agar Noble (Difco) 2% at pH 8.2 in 0.05 ionic strength barbital buffer or in cellulose acetate membrane using pH 8.6 barbital buffer solution of 0.075 ionic strength. Immunoelectrophoresis was performed according to SCHREIBER [11]. Analytical ultracentrifugation was performed by means of a Spinco Model E centrifuge at 52 000 rpm 27°C.

Isolation of pyroglobulins. Purified preparations were obtained by preparative electrophoresis in agar gel or in starch block. The γ -globulins collected were separated by passing them through Sephadex G 200 (2.5 × 100 cm Pharmacia) column using 1 M sodium chloride in 0.1 M tris HCl pH 8 or through a DEAE column starting buffer of 0.01 M phosphate pH 8 (final buffer of 0.3 M phosphate, pH 8).

Effects of chemical reagents on thermoprecipitability. Reduction and alkylation of serum or of isolated proteins were carried out with 0.1 M 2-mercaptoethanol and with dialysis against 0.02 M iodoacetamide. Other effects on thermoprecipitability were ascertained using dialysis against hypertonic solutions of NaCl (from 0.9 to 3%) or against phosphate buffers at pH ranging from 3 to 7.

Results

Characteristics of Eight Pyroglobulinemic Serums

The distribution of pyroglobulins among our paraproteinemic patients is summarized in table I. As can be seen, pyroglobulins are much more frequent in Waldenström's disease (6 cases out of 44), especially when associated with cryoglobulinemia (3 cases out of 5), than they are in myeloma (2 out of 216).

Of particular interest are the findings with regard to a case of IgA myeloma: only one case of an IgA pyroglobulin has been previously reported [12]. In table II, some clinical and serological data of our cases are given. The high sedimentation constant of cases S N, P E, C A is due to the associated mixed cryoglobulins, which results in circulating IgM-IgG complexes.

Pyroglobulin Characterization

(1) *Analysis of whole serum.* In all our cases, there was electrophoretic identity between pyroglobulin and paraprotein (fig. 1). This identity was confirmed by the loss of M-component from serum after precipitation of pyroglobulin (fig. 2).

(2) *Analysis of isolated pyroglobulins.* The electrophoretic, immunoelectrophoretic and ultracentrifugal characteristics of an IgG and IgA

Table 1. Incidence of pyroglobulins in our paraproteinemic patients

	Number of patients	Pyroglobulins
IgG Myeloma	151	1
IgA Myeloma	51	1
IgD Myeloma	2	
Bence Jones myeloma	12	
Waldenström's disease without cryoglobulinemia	39	3
Waldenström's disease with cryoglobulinemia	5	3
Total	260	8

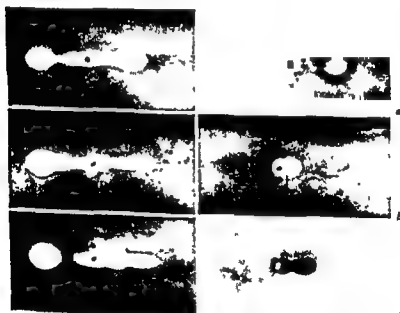


Fig. 1. After the employment of 3 pyroglobulin sera. On the left, the slides were fixed in 2% glutaraldehyde; on the right, identical preparations heated to 40°C without fixative. Case II, IgG myeloma; b, Case I, IgA myeloma; c, Case III, IgA myeloma; d, Case I, Waldenström's disease; e, Case II, Waldenström's disease; f, Case III, Waldenström's disease.

Table II Summary of clinical and

Patient	Sex	Age	Diagnosis	Cryoglobulins (cryocrit)	Free serum L-chain	Urine proteins B J
M L	M	59	myeloma	-	-	+
I R	M	55	macroglobulinemia	-	-	-
F A	M	60	macroglobulinemia	-	-	-
S N	F	61	macroglobulinemia	IgM IgG (20%)	-	-
D T L	F	66	macroglobulinemia	-	-	-
P E	F	44	macroglobulinemia	IgM IgG (15%)	-	-
C A	F	46	macroglobulinemia	IgM IgG (27%)	λ	+
M A	F	59	myeloma	-	λ	+



Fig 2 Cellulose acetate electrophoresis of serum M L (IgG myeloma) Whole serum (upper) the isolated M-component (middle) the supernatant after heating serum at 49°C (lower)

isolated pyroglobulins are shown in figures 3 and 4. As to the cases with associated cryoglobulinemia we found that pyroglobulins were recovered almost completely, in the redissolved cryoprecipitate. The isolation of the 2 components, IgM and IgG of the mixed cryoglobulins showed that pyroprecipitation depends exclusively on the IgM-component (fig. 5)

Laboratory features of patients

Patient sex and age	Total protein g%	M-Components			
		electrophoretic %,	heavy chain class	light chain type	sedimentation constant (S 20 W)
	11	71.67	IgG	κ	7
	11.4	71.61	IgM	κ	19
	10	71.44	IgM	λ	19
	19	71.52	IgM	κ	21
	8.7	71.26	IgM	κ	18
	8.4	71.37	IgM	κ	23
	10.8	71.44	IgM	κ	23
	9	6.30	IgA	κ	12.5

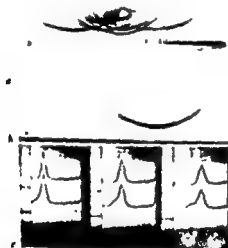


Fig. 1. Case M. M (IgG) (upper well) and isolated M-component (lower well) precipitation with rabbit anti-human serum. b Immunoelectrophoresis of isolated M component with an anti-human serum. c Analytical ultracentrifugation of 24, 32, and 48 hr, respectively, after reaching maximal peak for the isolated M-component. Lower

serum (upper well) and rabbit anti-human serum (lower well) precipitation.

(b) Effect of various reagents on pyroprecipitation was also presented in table III. The treatment of serum with acetamide did not inhibit the pyroprecipitation in most cases to cause spurious pyroprecipitation.

(c) Effect of such as acetamide, alcohol, and lower temperature on the

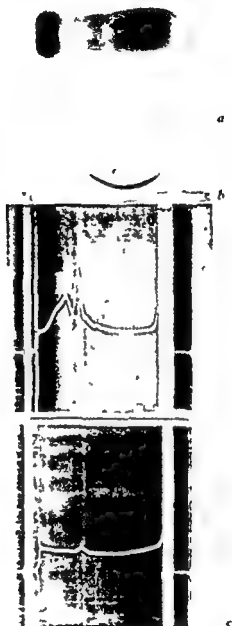


Fig. 4. Case M. A (IgA myeloma). a Cellulose acetate electrophoresis of serum and isolated M-component. b Immunoelectrophoresis of isolated IgA paraprotein in the trough rabbit anti-immunoglobulin serum. c Ultracentrifuge pattern. Upper whole serum, lower isolated IgA. Photographs taken at 32 min.



Fig. 1. Case 5-5 (Waldenström's disease with cryoglobulinemia). Gel electrophoresis of serum (upper) and isolated paraprotein (lower). A gamma band is observed in the upper gel, the bands of components (IgM IgG) of the mixture are in the lower gel. The purified IgM in the trough, as immunoelectrophoresis. Analytical ultracentrifugation of isolated IgM. Photographs taken at 6 and 24 hr after run at 92,000 rpm.

due to a breaking of H-bond bonds effected by the acidity of a favouring the successive action of heating. Only in a serum with IgM cryoglobulins we observed, in the same conditions, a delay in their sedimentation. We did not know the cause of this delay.

Table III. Effect of various conditions on precipitability of pyroglobulins at 60°C

Patient	Pyroglobulin	2 M Γ	pH 4	3% NaCl
M L	IgG _A	0	0	-
I R	IgM _A	0	-	-
I A	IgM _A	0	-	0
S N	IgM _A	0	+	-
D T L	IgM _A	0	+	+
P E	IgM _A	0	0	-
C A	IgM _A	0	+	-
M A	IgA _A	0	+	-

0 = No effect, + = increased pyroprecipitation, - = partial inhibition of pyroprecipitation

On the other hand, dialysis against hypertonic solutions partially inhibited the pyroprecipitation: the higher activity of hydrophobic groups in hypertonic solutions resulting in a greater stability of the molecule can explain this finding.

Discussion

Our results confirm previous reports on the close association between pyroglobulinemia and paraproteinemia. The incidence of this finding is about 2-3% (4 cases out of 162 in the series of COLLIER *et al.* [2], 8 out of 260 in ours), conversely we have found no cases without paraproteinemia. A possible explanation of the association is that pyroglobulins, normally present in serum in very low concentration, reach detectable serum levels only when an immunoproliferative disease affects the clone specifically committed to their synthesis, on the contrary can be conceived pyroglobulins as abnormal immunoglobulins absent in normal serum. A response to this question is strictly connected with the answer about the nature of the M-components.

Another interesting finding is the high frequency of the association between pyroglobulins and mixed cryoglobulins in Waldenström's disease (3 pyroglobulins out of 5 cases with cryoglobulins on a total of 44 cases of Waldenström's disease). These findings, already reported by MELTZER and FRANKLIN [6] (1 case) are particularly evident in our report.

In the present study we have demonstrated the identity of the molecule IgM responsible for the 2 phenomena. In both cases, only H-chains are engaged in the thermal reactions [1, 8]. Contrary to the results of PATTERSON *et al* [10], regarding the presence of both IgM and IgG in an IgM pyroglobulin precipitate dissolved in 2-mercaptoethanol, we have found that only the IgM, isolated from a mixed IgM-IgG-cryoprecipitate, was the pyroprecipitable component.

Moreover, we have investigated the molecular basis of pyroprecipitation. The different response from each pyroglobulin to the same physico-chemical agents does not support any conclusion. This variability is probably due to the various effects of thermal denaturation, giving rise sometimes to intermolecular complexes, at times active only inside the molecule.

PATTERSON *et al* [10] arrived at the same conclusion by observing a variable pattern in thermoprecipitation not only between pyroglobulins of different classes (IgM and IgG), but also of the same class (IgG). MORSE [7], studying the susceptibility to heat aggregation of isolated myeloma globulins, showed that 11 of 18 failed to aggregate independently of their electrophoretic mobility, thus suggesting a great variability in the degree of thermoreactivity between immunoglobulins. According to ZINSTEIN and STAL [13], the possibility of bringing into solution the pyroprecipitate by sodium dodecyl sulfate, an amphiphile, suggests that hydrophobic bonding is implicated in the mechanism of thermoprecipitation.

In our cases, the thermolability was not inhibited by 2-mercaptoethanol followed by butyramide. Similar result was observed by STAL [12] in the case of IgA pyroglobulin. This indicates that the formation of intermolecular disulfide bonds is not the only mechanism of precipitation.

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Table III. Effect of various conditions on precipitability of pyroglobulins at 60°C

Patient	Pyroglobulin	2 ME	pH 4	3% NaCl
M L	IgG λ	0	0	-
I R	IgM λ	0	-	-
F A	IgM λ	0	-	0
S N	IgM λ	0	+	-
D T L	IgM λ	0	+	+
P E	IgM λ	0	0	-
C A	IgM λ	0	+	-
M A	IgA λ	0	+	-

0=No effect, +=increased pyroprecipitation -=partial inhibition of pyroprecipitation

On the other hand, dialysis against hypertonic solutions partially inhibited the pyroprecipitation: the higher activity of hydrophobic groups in hypertonic solutions resulting in a greater stability of the molecule can explain this finding.

Discussion

Our results confirm previous reports on the close association between pyroglobulinemia and paraproteinemia. The incidence of this finding is about 2-3% (4 cases out of 162 in the series of COLLIER *et al* [2], 8 out of 260 in ours), conversely we have found no cases without paraproteinemia. A possible explanation of the association is that pyroglobulins, normally present in serum in very low concentration, reach detectable serum levels only when an immunoproliferative disease affects the clone specifically committed to their synthesis, on the contrary can be conceived pyroglobulins as abnormal immunoglobulins, absent in normal serum. A response to this question is strictly connected with the answer about the nature of the M-components.

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Table III Effect of various conditions on precipitability of pyroglobulins at 60 C

Patient	Pyroglobulin	2 M Γ	pH 4	3% NaCl
M L	IgG λ	0	0	-
I R	IgM λ	0	-	-
F A	IgM λ	0		II
S N	IgM λ	0	+	-
D T L	IgM λ	0	+	+
P F	IgM λ	0	0	
C A	IgM λ	0	+	-
M A	Ig λ λ	II	+	-

0 = No effect + = increased pyroprecipitation, - = partial inhibition of pyroprecipitation

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Viskositätsuntersuchungen mit Fibrinogenspaltprodukten

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Zusammenfassung. In *in vitro* Untersuchungen using the Harkness viscometer a logarithmic increase of the viscosity of Fibrinogen degradation could be demonstrated for increasing concentrations of Fibrinolytic the rugen degradation products (FDP). This increase of Viscosity viscosity could also be observed with increasing concentrations of FDP plus Fibrinogen. However, increasing concentrations of Fibrinogen showed a linear increase of viscosity. In testing concentrations of FDP plus gelatin, we showed a linear curve in contrast to the exponential increase of viscosity of the rugen plus gelatin.

Additionally, FDP showed a lower viscosity in contrast to Fibrinogen of a comparable viscosity using a Bink blood viscometer at different shear rates, in presence of a suspension of erythrocytes.

Als Faktoren der intravasalen Mikrozirkulation gelten neben Perfusionsdruck und Gefäßdurchmesser die Blut- und Plasmainviskosität, Aggregationsneigung und Formveränderung der Erythrozyten, Thrombozytenaggregation und Gerinnungsprozesse wie Verbrauchthrombolyse und Fibrinolyse [1-10]. Störungen der Mikrozirkulation hängen daher eng von Veränderungen dieser Parameter ab.

Genauere Untersuchungen über den Zusammenhang und die Abhängigkeit der Viskosität und Plasmainviskosität vom Fibrinogenspaltprodukten (FDP) wurden bisher nur durch Schmitt

* Mit Unterstützung der Bundesministerien für Forschung und Technologie und für die Wissenschaften.

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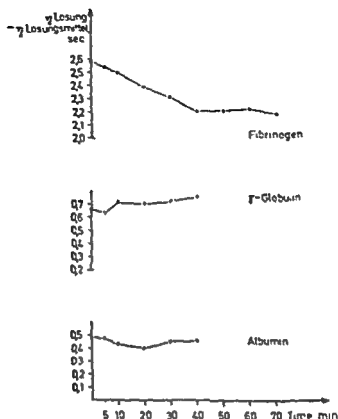
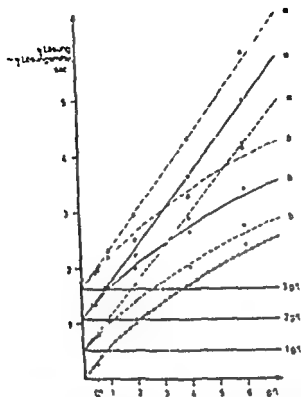


Abb. 1 Die Einwirkung von Streptokinase (je 100 E/ml) auf Fibrinogen γ -Globulin und Albumin

Material und Methodik

Die Viskosität der Fibrinogen- γ -Globulin- und FDP-Lösungen wurden mit dem Harkness-Viskosimeter [5] gemessen, das einen konstant reproduzierbaren Schergrad von 660 sec^{-1} einen konstanten Druck von 75 mm Hg und eine kinematische Viskosität von $2 \cdot 10^{-2} \text{ cm}^2 \text{ sec}^{-1}$ hat. Die Ableseung erfolgte durch elektrische Zeitmessung mit einer Genauigkeit von 0,02 sec als Durchschnittswert von 5 Einzelmessungen. Die Angabe der Viskosität erfolgte in Sekunden als relativer Wert zum Lösungsmittel ($\eta_{\text{Lösung}} / \eta_{0,9\% \text{ige NaCl}}$). Die Viskosität der Erythrozytensuspension wurde mit einem Wells-Brookfield-Viskosimeter Modell IVT, bei den Schergraden 115, 46, 23 und $11,5 \text{ sec}^{-1}$ bei 37°C gemessen [12]. Begonnen wurde beim Schergrad 115 sec^{-1} , gewartet wurde für etwa 2–3 min und zwischen jeder Messung die Suspension durch Kippen des Tellers neu vermischt.

Die Untersuchungen wurden mit Fibrinogen (Forschungs-Fibrinogen-Kabi-Deutsche Kabi GmbH München 15), γ -Globulin (γ -Globulin aus Humanserum, Lymphophil, rein 98%, Behringwerke AG Marburg/Lahn) sowie Streptokinase (Streptase® Behringwerke AG Marburg/Lahn), Amino-capronsäure (FACA Behring

[illegible]

Wesentliches Merkmal: Nach einer Zeit konnte keine weitere Vinkovits-
Anpassung gemessen werden (Abb. 1). Albumin und -Globuline zeigten
eine typische Konzentrationswirkung keine Vinkovits-Anpassung (Abb. 1).

Der Verlauf der Abgabe beim Ethernogen unter Streptokinaseeinwirkung hängt von der Ausgangskonzentration und Ausgangspunkten ab und ist um so ausgeprägter, je höher diese anfangs waren (Abb. 2). Die gleiche pro Gabe von 1 ACA + Streptokinase zu Ethernogen verhindert den bei 200 pro Gabe von Streptokinase beobachteten Rückgang der Abgabe während des 4. und 5. Tages (Abb. 2 und 3). Die gleiche pro Gabe

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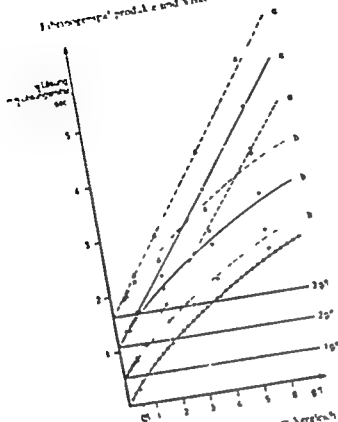
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1. Hydrogen peroxide and formalin

[illegible]

über nicht abnehmend. Nach dieser Zeit konnte keine weitere Volumen-
änderung gemessen werden (Abb. 1). Albumin und -Globuline zeigten
ein starkes Absinken während keine Volumenänderung (Abb. 1).
Die Volumenänderung beim Erhitzen einer Streptokokinase war
hängt von der Ausgangskonzentration und Ausgangspunkt ab.
Wie man aus Abb. 1 und 2 sieht, so ist die Streptokokinase zu Erhitzen verbunden
sich mit der Erhitze von 100°C - Streptokokinase zu Erhitzen verbunden
den bei 100°C Erhitze von 100°C - Streptokokinase zu Erhitzen verbunden
Volumen während Erhitzen (Abb. 2 und 3). Die Streptokokinase

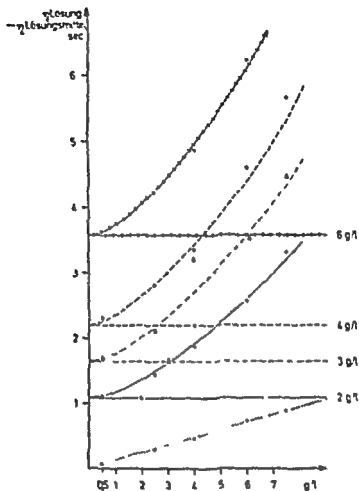


Abb 5 Viskositätsverhalten von Fibrinogen und γ -Globulinen in unterschiedlichen Konzentrationen Konstant Fibrinogen — + — = 2 g/l, --- Δ --- = 3 g/l, -- \bullet -- = 4 g/l, $\dots \times \dots$ = 6 g/l Variabel γ -Globulin (0.5, 2.5, 4, 6 und 7.5 g/l, + = γ -Globulin (Einzelmessung)

von Trasylol + Streptokinase zu Fibrinogen bewirkt dagegen keine Viskositätsänderung. Eine Hemmung der Streptokinaseeinwirkung lässt sich daher *in vitro* nur durch Trasylol, nicht durch EACA durchführen (Abb 3). Eine Eigenwirkung des Trasylols bzw. der EACA auf die Viskosität kann ausgeschlossen werden (Abb 3).

Der Viskositätsverlauf unterschiedlicher Fibrinogenkonzentrationen zeigt ein additives Verhalten und einen linearen Verlauf (Abb 4). Der Viskositätsverlauf einer FDP-Lösung aus unterschiedlichen Konzentrationen

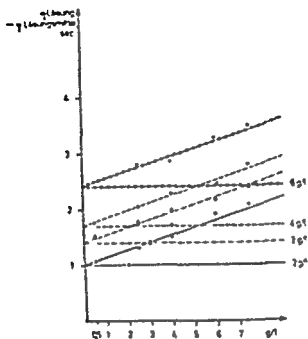


Abb. 4. Viskositätsverhalten von FDP und γ -Globulin mit unterschiedlicher Konzentrationen. Viskosität FDP (aus 2, 3, 4 und 6 g/l Fibrinogen nach Sjöqvist) relativ zu einem $\eta(100) = 10$ (in mN) = —•—•— 2 g/l = —Δ—Δ— 3 g/l, —○—○— 4 g/l = + + + + + 5 g/l. γ -Globulin-Konzentration 10, 5, 2, 3, 4, 6 und 7,5 g/l.

ren einer Fibrinogenlösung durch Streptokinase hergestellt, zeigt einen logarithmischen Verlauf (Abb. 4). Der Viskositätsverlauf unterschiedlicher Fibrinogenkonzentrationen mit unterschiedlichen FDP zeigt ebenfalls ein additives Verhalten, jedoch einen logarithmischen Kurvenverlauf (Abb. 4). Der Viskositätsverlauf unterschiedlicher Fibrinogenkonzentrationen mit unterschiedlichen γ -Globulin-Konzentrationen zeigt im Gegensatz zu reinem γ -Globulin (Abb. 5) und reinem Fibrinogen (Abb. 4) ein additives Verhalten und einen exponentiellen Verlauf (Abb. 5). Der Viskositätsverlauf unterschiedlicher FDP-Konzentrationen mit unterschiedlichen γ -Globulin-Konzentrationen zeigt ebenfalls ein additives Verhalten, jedoch einen linearen Verlauf (Abb. 6). Eine Extrapolationsspannung der FDP oder Fibrinogen gleich der Viskosität zugegeben wurde, zeigt mit FDP behandelte Schlangenblutsera eine niedrige Viskosität (Abb. 7).

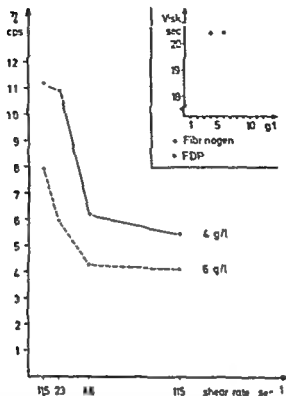


Abb 7 Verlauf der Viskosität einer Erythrozytensuspensionslösung der Fibrinogen oder FDP gleicher Viskosität zugegeben wurden —+—=4 g/l Fibrinogenlösung mit Erythrozytensuspension (Hämatokrit $40 \pm 1\%$, 37°C) --+--=6 g/l FDP Lösung nach Streptokinaseinkubation (mit 100 E/ml 60 min) mit Erythrozytensuspension (Hämatokrit $40 \pm 1\%$, 37°C)

Diskussion

Viskositätswirksame Parameter für die Plasmapviskosität sind das Fibrinogen und die γ -Globuline und für die Vollblutviskosität zusätzlich der Hämatokrit und die Erythrozytenaggregationstendenz [7]. Besonders Wechselwirkungen des Fibrinogens mit den γ -Globulinen und den Erythrozyten sind hierfür verantwortlich, und für Veränderungen der Viskosität werden daher vorwiegend Konzentrationsänderungen des Fibrinogens angeführt [3, 8]. Besonders eindrucksvoll konnte dies an therapeutischen Fibrinolyse, die mit einer Fibrinogenolyse einhergehen, demonstriert werden [1, 2, 4].

Trotzdem sollten die bei unterschiedlichen Krankheiten und besonders bei fibrinolytischen Zuständen anfallenden FDP [6] hinsichtlich ihrer Vis-

Im Zusammenhang untersucht werden. Dabei konnten wir aufgrund unserer Untersuchungen einen wesentlich geringeren viskositätswirksamen Effekt der bei einer Fibrinogenolyse aus dem hochmolekularen Fibrinogen entstehenden niedermolekularen Spaltprodukte D und E [11] sowohl auf das Fibrinogen und die γ -Globuline als auch auf eine Erythrozytensuspension feststellen. Klinisch bedeutet dies, dass bei Krankheitsbildern, die mit dem Auftreten besonders hoher Konzentrationen von FDP einhergehen [6] die Plasmainviskosität sowie ihr Einfluss auf die Vermittlungsviskosität sich anders verhält als bei Krankheiten ohne FDP, aber etwa gleicher Fibrinogen-, γ -Globulin- und Hämatokritkonzentration.

Zusammenfassung

In viskositätsmessungen mit dem Harkness-Viskosimeter konnte für ansteigende Konzentrationen von Fibrinogen-Spaltprodukten (FDP) ein logarithmischer Viskositätsanstieg gezeigt werden. Dieser logarithmische Viskositätsanstieg trat ebenfalls bei ansteigenden Konzentrationen von FDP plus Fibrinogen auf, während ansteigende Fibrinogenkonzentrationen allein einen linearen Viskositätsanstieg zeigten. Ansteigende Konzentrationen von FDP plus γ -Globulinen zeigten im Gegensatz zum erwarteten Viskositätsanstieg von Fibrinogen plus γ -Globulinen einen linearen Verlauf. Außerdem zeigten am Brookfield-Viskosimeter bei unterschiedlichen Scherspannen FDP im Gegensatz zum Fibrinogen gleicher Ausgangskonzentration mit einer 10- bis 20-fachen Viskositätssteigerung niedrigere Viskosität.

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Binding of Folic Acid to Serum Proteins

I. The Effect of Pregnancy

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Abstract. Binding of serum folic acid activity (FAA) to the protein fraction during and after pregnancy was studied by DEAE Sephadex A 40 gel chromatography. Towards the end of pregnancy this binding increased. The increase was particularly distinct in the binding to transferrin. At the same time the binding to α_2 macroglobulin decreased. Within a week after delivery the total protein binding of FAA decreased remarkably. The decrease was also particularly distinct in transferrin.

Key Words

Carrier proteins of folic acid
Folic acid metabolism
Gel chromatography
Pregnancy
Serum proteins

In our laboratory it could be shown that just under half of the serum folic acid activity (FAA) is bound to various proteins [8, 7, 9]. Some FAA travel with albumin [4], a finding that has been corroborated elsewhere [2]. Usually the majority of the bound FAA is filtered in chromatography with α_2 macroglobulin [10] and the balance with transferrin [11]. These results have been obtained from fresh blood specimens of healthy test subjects. Owing to bacterial contamination, however, the chromatography had to be carried out in cold conditions at -10°C . and in the dark, and it is naturally possible that temperature might affect the binding of FAA with proteins. In the course of a fairly long follow-up period the chromatography results obtained from one and the same person has always been very much the same (unpublished observations).

Since the normal chromatographic distributions of FAA have now been determined in this way, it is interesting to study how the different factors physiologically or pathologically affect the normal FAA distribution. Pregnancy is known to be a factor which considerably changes the total

homeostasis of serum FAA. Some results of a preliminary study of the influence of pregnancy on the binding of FAA to serum proteins are presented in the following.

Material and Methods

The changes in the binding of serum FAA during and after pregnancy were followed in 2 primiparas by chromatography. Detailed characteristics can be seen from table I. The examined women were in good health and ingested Finnish staple food without additives throughout the period of observation. For the present study they attended a special examination and underwent laboratory tests after the 20th (chromatography A) and the 30th (chromatography B) week of gestation and again 3 days (chromatography C) and 8 weeks (chromatography D) after normal delivery.

Chromatographies were made on absolutely fresh venous blood specimens. The treatment of the specimens and the chromatography techniques have been described in detail before [10]. Protein determinations were carried out using the biuret reaction and the FAA determinations with the L-cysteine method in the way described earlier [10].

Results

Figure 1 presents the chromatogram of a non-pregnant healthy young woman's serum. FAA is hardly seen at all in the first protein peak (γ -globulin). The second peak (γ -globulin and transferrin) shows 22% of the bound FAA, the third (the flat intermediate zone, transferrin) 10% and the fourth peak (α -2-macroglobulin) 30% of the bound FAA. The remainder of the FAA is scattered over the albumin area. Of the total FAA of the serum, 26% is bound to the protein area.

A review of both young women's chromatograms during pregnancy (fig. 2-3) shows that the A chromatogram (20th week of gestation) is very similar to that of a non-pregnant woman. Table I reveals, however, that the FAA binding percentage to serum proteins in the pregnant woman already is increased in this stage. The B chromatograms of the pregnant woman (30th week of gestation) disclose a violent increase in FAA binding: for in both test persons almost all serum FAA is bound to proteins, and the binding rate per protein unit has doubled (table I). The FAA increase in the transferrin area is particularly conspicuous: patient T V showed an increase from 45 to 50% and patient P T from 45 to 78%.

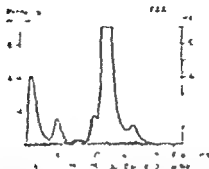


Fig. 1. Chromatogram of the serum of a healthy non-pregnant woman. Vertical lines indicate FAL against the background of the protein chromatogram, in which the first peak contains γ -globulin, the second α -globulin and transferrin, the third (flat) intermediate area transferrin, the fourth α_2 -macroglobulin, and the fifth β -globulin. The total FAL in the serum is 4700 μ g/ml, of which 27% are bound to proteins.

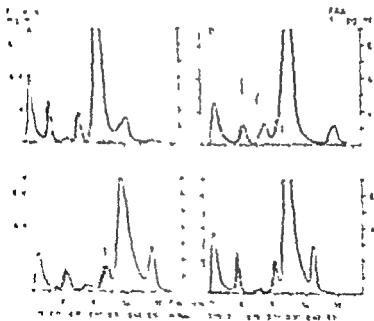


Fig. 2. The pregnant and lactating FAL in serum. A and C are chromatograms of the FAL of pregnant women, B and D after delivery. Note the increased binding of FAL to serum proteins in the second and third prenatal peaks.

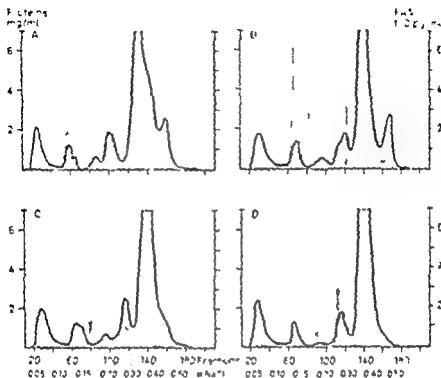


Fig. 2. The pregnant test subject P.T. 1 (for details see table I). As in figure 2 chromatogram B reveals a distinct increase in FAA in the area of the second and third protein node. Chromatogram C shows the disappearance of 1AA from the corresponding areas.

The amount of 1AA bound to α -2-macroglobulin has in both cases fallen by 15–20% compared with the earlier date of pregnancy (chromatography A).

Figures 2 and 3 and table 1 reveal, further, that a heavy fall has taken place in the protein binding of 1AA immediately after delivery (chromatography C). The fall took place mainly in the transferrin area, but some FAA has disappeared also from the albumin area. The change can be considered the exact opposite of the one taking place as pregnancy advanced. The fall in FAA binding was particularly violent in test subject P.T., not less than from 61 to 17 ng/g (table I). At the same time, it could be ascertained that no essential changes occurred in the protein content of the serum around the time of delivery. Also, the total FAA of the serum had remained approximately on the level recorded during pregnancy.

Table 1. Data and information on 2 pregnant test subjects at each chromatography

Test person	Time of chromatography	Hb g%	Wt g	Total FAA mg ml	Serum		
					protein mg ml	FAA protein-bound FAA percentage	percentage
Delivery after 37 weeks of pregnancy	A	105	672	39	636	304	47
	B	105	629	40	616	112	92
	C	121	575	27	648	172	41
	D	120	515	18	664	261	92
Delivery after 35 weeks of pregnancy	A	117	637	27	656	316	75
	B	120	530	40	664	651	100
	C	123	695	27	672	341	50
	D	117	550	31	676	250	37

* A after 37 B after 35 weeks of gestation C 3 days and D 2 weeks after delivery
 Test subject P T was 20 years old and the other 35 with the age was 2640 g Test subject
 P T was 27 years old and the other 35 with the age was 2640 g

The chromatography C figures (3 weeks after delivery) show that the late phase of lactation the protein binding of FAA begins to return to the normal proportions. The binding in this phase perhaps still exceeds that in a non pregnant woman. During lactation, test subject P T (fig. 3) developed a distinct FAA deficiency and almost all of her serum FAA is that the bound to proteins. In this phase of lactation no bound FAA at all is found in the albumin area. This was never the case with the non pregnant woman nor with either of the test subjects in the different phases of pregnancy.

Discussion

On the basis of this preliminary study we may draw the conclusion that the binding of FAA to proteins in the maternal serum increases during pregnancy. A particularly striking increase takes place in the transfer of FAA to albumin. This albumin-bound FAA appears and at the same

On the basis of our earlier studies, we have reported that in a healthy human individual the serum FAA, examined by chromatography, is eluted from the column carried by albumin, α -2-macroglobulin and transferrin [8, 10-11]. Very infrequently FAA is seen to be bound to γ -globulin (the first protein peak in DEAE Sephadex chromatography) [our unpublished finding]. Naturally, this gives rise to the question as to why FAA should have so many binders or carrier proteins. The kinetics of FAA have not as yet been extensively studied. The series of studies now inaugurated will, it is hoped, provide some tentative suggestions in this problem.

According to the preliminary studies, chromatography carried out under identical experimental conditions and in particular, in exactly the same way, always gives the same result for any one test person, both as regards the distribution of proteins and that of FAA [our unpublished finding]. The repeatability of the chromatography result is in our opinion extremely good. Hence, the change in FAA binding during pregnancy may probably be considered reliable. So far the physiological role of the change described is not known, nor are the factors that produce it known. As pregnancy advances, the protein content of blood per volume unit decreases, with the result that albumin decreases, α -1-, α -2-macroglobulin and transferrin increase, and β -2- and γ -globulin remain practically constant [12]. Similar changes were also observable in the present study, however, the relative increase in FAA per protein unit was very much more definite. It seems that, towards the end of pregnancy, FAA accumulates especially in transferrin from which it disperses immediately after delivery (into the mother's milk?). At the same time, the FAA which has been bound in the albumin area disappears. For α -2-macroglobulin, no particularly pronounced changes seem to take place either way. It might be assumed that albumin and transferrin are, in FAA metabolism, first degree carrier proteins and that the α -2-macroglobulin is a second-degree carrier which retains the FAA stores to the very last moment. In this connection it should also be noted that the above 2 components (albumin and transferrin) are synthesized in the liver.

According to a great deal of the literature, serum FAA decreases during pregnancy, and this may lead to the development of a deficiency state. On the basis of the present study it may be deduced that much greater changes perhaps take place in the internal distribution of serum FAA than in the total serum FAA. For this reason the aim of future research should, first and foremost, be to study the possible physiological factors

which produce the change in the distribution (the roles of the various hormones, liver, etc.) [1, 3-4] and the reasons why such a change takes place towards the end of pregnancy. Does it ensure an adequate FAA content of mother's milk? Does it retain FAA for the mother's haematopoietic needs? Or are there several simultaneous contributory factors? In any case, it is apparent that the binding of FAA to serum proteins decreases relatively soon after the delivery. The preliminary studies have shown that the prolonged use of hormonal contraceptives produces the changes described here as having taken place during pregnancy (our unpublished finding).

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Spezifische Mikrogranula in Eosinophilen

Eine vergleichende elektronenmikroskopische Untersuchung an verschiedenen Säugern zur Charakterisierung einer besonderen Granulationsform bei eosinophilen Granulozyten¹

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Abstract Besides the well known eosinophilic granules mature eosinophil granulocytes are containing a special form of smaller granules measuring about 100 (20-200) nm. The constant occurrence of these specific microgranules (SM) of eosinophils, as they are named is proved in eosinophils of 20 different mammalian species out of 7 orders including man. SM are composed of an opaque core bound with an unit membrane. SM may be distinguished into 3 varieties, a spheric (double spheric) a dumbbell and a cup type. The occurrence of one or the other type depends on the species. SM are supposed to be an essential cytoplasmic detail of mature eosinophils hitherto widely disregarded.

Key Words

Comparative hematology
Electron microscopy
Eosinophil granulocytes
Specific microgranules
Ultrastructure
of eosinophils

Bei früheren Untersuchungen [21] zur Alterung eosinophiler Granulozyten fielen in reifen Eosinophilen der Ratte elektronenmikroskopisch runde, granulumartige Gebilde auf, die sich von den bekannten eosinophilen Granula durch abweichende Struktur und weit geringere Dimensionen unterschieden. Solche Körper sind in der bisherigen Literatur zur Ultrastruktur der Eosinophilen weitgehend unbeachtet geblieben oder nur sporadisch als grössenordnungsmässig etwa 100 nm messende Gebilde in Eosinophilen einiger Laboratoriumstiere erwähnt worden [11, 16, 17, 19, 24]. Unsere Untersuchung hat zum Ziel, an einem möglichst breiten Spektrum verschiedener Säugerarten zu prüfen, ob solche Mikrogranula als regelmässiges und obligates Element der zytoplasmatischen Differenzierung reifer eosinophiler Granulozyten aufgefasst werden können, ob

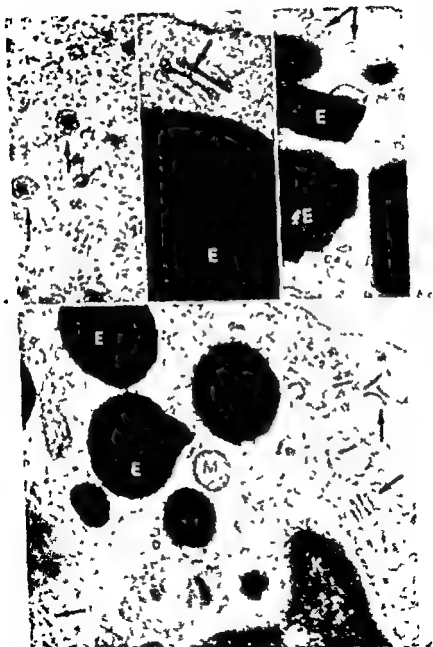
¹ Mit freundlicher Unterstützung der Deutschen Forschungsgemeinschaft

gierten Blutpräparation. Ihre Identifizierung und Abgrenzung insbesondere von Bluteosinophilen war durch den Vergleich mit den orientierten den lichtmikroskopischen Befunden in Ausstrichen und Semidünnschnitten stets zweifelsfrei möglich. Struktur und Grösse der eosinophilen Granula (EG) wiesen bei Mensch und Tier die bekannten artabhängigen Differenzen auf. Soweit besondere bisher unbekannte Formen von EG gefunden worden sind, soll deren Beschreibung gesondert erfolgen.

Der Nachweis *spezifischer Mikrogranula* (SM) gelang in den reifen Bluteosinophilen aller untersuchten Spezies. In jeder Blutpräparation wurden mindestens 25 Eosinophile mikroskopiert, welche sämtlich SM enthielten. Lediglich bei den Tigern konnten nur 5 Eosinophile pro Tier aufgefunden werden (Stresseosinopenie nach längerer Erregung und Gegenwehr beim Fang der Tiere mit Netzen), die aber ebenfalls alle SM enthielten. In ihrer einfachsten Form bilden SM runde Kugeln in einer Größenordnung von 100 nm (Abb. 1a, 3). Ihr Inhalt ist verglichen mit dem umgebenden Grundzytoplasma relativ elektronendicht, er wird von einer Einheitsmembran umgeben, die bei höherer Auflösung eine typische Dreischichtung zeigt. Dieser einfachste Typ ist prototypisch bei der Wanderratte entwickelt und kommt auch bei den meisten übrigen Mäusen vor (Tab. 1 Nr. 6, 12). Nicht selten finden sich neben einfachen auch Doppelkugeln.

Von diesem Typ der Doppelsphäre leitet sich durch Längsdehnung ein weiteres hantelförmiges Schnittprofil ab, wie es typisch bei Hund (Abb. 1d), Pferd (Abb. 1b) und etwas plumper gestaltet auch beim Menschen (Abb. 2) und einigen anderen Arten ausgeprägt ist (Tab. 1). Abgesehen von ihrer besonderen Form unterscheiden sich diese flacheren Mikrogranula durch ihren dichteren Inhalt von den Resten des bei reifen Eosinophilen weitgehend zurückgebildeten endoplasmatischen Retikulum, dessen Zisternen – am besten sichtbar am Beispiel der perinuklearen Zisterne (Abb. 1d, 2) – transparent erscheinen. Hantelförmige SM stellen gestreckte Profile dar, die z.B. beim Pferd in der Länge 220 nm in der schmalen Mittelzone 20 nm messen. Da bei den SM dieses Types offen-

Abb. 1. Spezifische Mikrogranula (Pfeile) in eosinophilen Granulozyten (E = eosinophile Granula, M = Mitochondrien, K = Kern mit z.T. tangential getriebener perinukleärer Zisterne) verschiedener Tierarten: Kugeltyp bei der Ratte (a), Hanteltyp beim Pferd (b), Napftyp mit U- und O-Präfilien bei der Katze (c) und Übergänge zwischen Hantel- und Napftyp beim Hund (d). Man beachte die unterschiedlichen Endvergrößerungen bei a c etwa 60.000 \times b d etwa 30.000 \times .



gierten Blutpräparation. Ihre Identifizierung und Abgrenzung insbesondere von Bluteosinophilen war durch den Vergleich mit den orientierenden lichtmikroskopischen Befunden an Ausstrichen und Semidünnschnitten stets zweifelsfrei möglich. Struktur und Grösse der eosinophilen Granula (EG) wiesen bei Mensch und Tier die bekannten artabhängigen Differenzen auf. Soweit besondere, bisher unbekannte Formen von EG gefunden worden sind, soll deren Beschreibung gesondert erfolgen.

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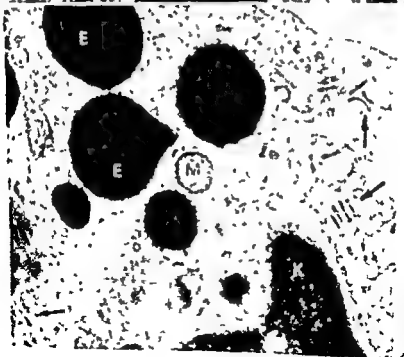


Tabelle 1 Form und Grösse spezifischer Mikrogranula in Eosinophilen bei Menschen und einigen Säugetieren

Ordo und Spezies	Formtyp	Mittlere Grösse nm
I Marsupialia		
1 <i>Marmosa murina</i>	Napf (Hantel)	80 160
II Insectivora		
2 <i>Erinaceus europaeus</i>	Hantel	70 150
3 <i>Hemiechinus auritus</i>	Hantel	140 290
III Rodentia		
4 <i>Sciurus vulgaris</i>	Hantel	20 220
5 <i>Ondatra zibethica</i>	Hantel	15 200
6 <i>Arvicola terrestris</i>	Kugel	70
7 <i>Micromys minutus</i>	Kugel	45
8 <i>Rattus norvegicus</i>	Kugel	90
9 <i>Proomys morio</i>	Kugel	70
10 <i>Proomys tullbergi</i>	Kugel	100
11 <i>Acomys cahirinus</i>	Kugel	60
12 <i>Acomys minous</i>	Kugel	60
13 <i>Cavia porcellus</i>	Hantel	20 160
14 <i>Myocastor coypus</i>	Hantel	30 190
IV Carnivora		
15 <i>Canis familiaris</i>	Hantel (Napf)	20 210
16 <i>Felis catus</i>	Napf	20 200
17 <i>Panthera tigris</i>	Hantel (Napf)	20 240
V Lagomorpha		
18 <i>Oryctolagus cuniculus</i>	Hantel	25 180
VI Perissodactyla		
19 <i>Equus caballus</i>	Hantel	20 220
VII Primates		
20 <i>Homo sapiens</i>	Hantel	20 210

Abb 2 Spezifische Mikrogranula (Pfeile) in einem reifen Eosinophilen des Menschen (F eosinophile Granula K Kern in Bldmitte tangential angeordnet mit heller perinuklearer Zone M Mitochondrien) Vergrößerung etwa 42 000fach



bar verschiedene Anschnittwinkel relativ konstant und ohne wesentliche Formabweichungen ein Hantelprofil produzieren, dürfte die räumliche Gestalt einer zentral bikonkav eingedellten Scheibe entsprechen, ähnlich etwa der Form eines Erythrozyten. Mit roten Blutkörperchen haben solche SM auch eine Tendenz zu geldrollenartiger Stapelung gemeinsam (Abb 1b). Dieses Verhalten kann durch eine gewisse Adhäsionswirkung der Scheibenflächen erklärt werden.

Der Hantel- oder Scheibentyp leitet durch tassenförmige Einstülpung der Scheibe zu einer dritten, napfförmigen Variante über, die prototypisch bei der Hauskatze vorkommt. Je nach dem Anschnittwinkel der napfförmigen SM resultieren gewölbte U- oder ringförmige O-Profile (Abb 1c).

Bei manchen Arten kommen besonders die Scheiben- und Napfformen nebeneinander vor (Abb 1d). Bemerkenswert ist jedoch eine grundsätzliche Artabhängigkeit und Artkonstanz der verschiedenen Formtypen und des Spielraumes ihrer Dimension. In ihren Extremen (z.B. SM von Ratte, Pferd und Katze) unterscheiden sie sich daher augenfällig (Abb 1a-c).

Von EG unterscheiden sich SM schon durch ihre mindestens um den Faktor 10 geringere Grösse, wobei Übergangsformen fehlen. Von Sekundärgranula in Neutrophilen lassen sich SM durch ihre Form abgrenzen, sie haben mit diesen zwar eine vergleichbare Dimensionierung gemeinsam, eine Isomorphie besteht aber nicht oder ist allenfalls bei einigen Arten angedeutet.

In eosinophilen Vorstufen des Knochenmarks fehlen SM stets. Auf die Gesamtzahl aller Mark eosinophilen bezogen schwankt die Häufigkeit ausgereifter und SM-haltiger Eosinophiler zwischen 5 und 10% (Anzahl mikroskopierter Eosinophiler pro Präparation mindestens 50). Bemerkenswert ist auch das häufige Fehlen von SM in scheinbar reifen Mark eosinophilen, während sie in Eosinophilen aus dem Blut oder in Gewebe eosinophilen (Rattenuterus: Anzahl mikroskopierter Eosinophiler pro Uterus mindestens 50) regelmässig anzutreffen sind. Das gilt auch für reife Bluteosinophile von Fällen chronischer Myelose oder akuter Myelose mit Begleiteosinophilie beim Menschen. Offenbar bilden SM ein terminales

Abb 3 Wandernder Eosinophiler im oestrogenstimulierten Rattenuterus. Pseudopodienartige Zytoplasmafortsätze enthalten nur spezifische Mikrogranula (Pfeile). eosinophile Granula (F) sammeln sich in Nähe des Kernes (K) an. M = Mitochondrien, z.T. regressiv veränderte Endvergrößerung etwa 18 000fach.



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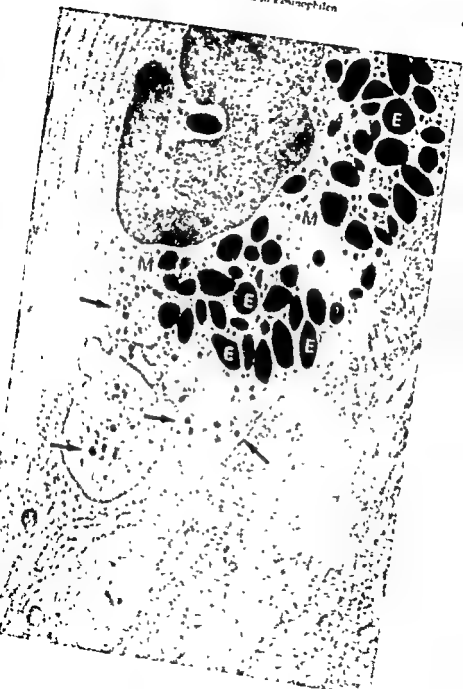
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Differenzierungsprodukt aus der Phase des Überganges vom stabkernigen zum segmentkernigen Granulozyten. Gelegentlich scheinen sich Vesikeln des in Rückbildung begriffenen Golgiapparates in SM umzuwandeln, die genaue Entstehung bedarf aber noch weiterer Klärung.

Aus der Bluthahn in das Gewebe ausgewanderte und dort lokomotorisch aktive Eosinophile, wie sie unter Östrogeneinfluss im Rattenuterus auftreten, zeigen oft eine besondere Verteilung ihrer Granulation. Während sich EG in der kernnahen Zytoplasmazone versammeln bevorzugen SM periphere Zytoplasmakompartimente und sammeln sich dort gern in pseudopodienartigen Fortsätzen an (Abb. 3). Diese örtliche Dissoziation von EG und SM stellt ein Charakteristikum des vermutlich funktionell aktiven Gewebeeosinophilen dar. Bluteosinophile und diejenigen durch eine mehr homogene Verteilung von EG und SM gekennzeichnet.

Diskussion

Der Nachweis eines konstanten Vorkommens von SM in reifen Eosinophilen bei 20 Säugetierarten aus 7 verschiedenen Ordnungen (Tab. I) macht wahrscheinlich, dass es sich um ein zytoplasmatisches Differenzierungsprodukt handelt, dem ungeachtet der möglichen funktionellen Bedeutung eine ähnliche Wertigkeit zukommt wie den seit FÄHRLEICH [10] bekannten EG. Wegen ihrer geringen Grösse sind SM nur elektronenmikroskopisch sichtbar, so dass sie von der klassischen (lichtmikroskopischen) Hamatomorphologie nicht erfasst worden sind. Aber auch elektronenmikroskopisch sind SM zumeist übersehen worden, teils aus Gründen noch mangelhafter Technik, teils aus einer vorwiegenden Fixation des Interesses auf die grösseren EG.

Nediglich einige Autoren haben SM (bzw. entsprechende Gebilde) in Eosinophilen von Laboratoriumtieren beiläufig erwähnt. So beschreibt PALADE [16] bei Ratteneosinophilen rundliche Körper, die er als besondere Ausprägungsform des glatten endoplasmatischen Retikulum auffasst. POLICARD *et al.* [17] erwähnen bei der gleichen Tierart besonders in Gewebeeosinophilen anzutreffende « *fines vesicules de tailles variables* » während später ROSS und KIRBY [19] am gleichen Beobachtungsobjekt ohne weiteren Kommentar von « *small round granules* » sprechen. Offenbar ebenfalls unseren SM entsprechende « *smaller granules* » führten GREY und BILSELF [11] in der Abbildungslegende eines Eosinophilen der Maus auf.

Neutrophilen haben WARD *et al.* [24] sogenannte Sekundärgranula in Eosinophilen der Katze beschrieben die in Form und Grösse den SM entsprechen und übrigens auch vollkommen mit sogenannten C Granula übereinstimmen welche WATANABE [25] bereits 1957 in Eosinophilen der Katze abgebildet hat und die zumindest grössenordnungsmässig identisch sind mit vergleichbaren Gebilden die ebenfalls in Katzeneosinophilen von HARTMANN und KNOOR [6] als «Mikrogranula» kurz erwähnt worden sind. Die Bezeichnung «Sekundärgranula» soll andeuten, dass diese Granula als 2. Generation analog der Bildung von Sekundärgranula in Neutrophilen auftreten.

Bekanntlich sind in der neutrophilen Granulogenese im Promyelozytenstadium gebildete Primärgranula von den vorwiegend im Myelozytenstadium gebildeten Sekundärgranula unterschieden worden [9, 26, 27], die letzteren sind nach den autorisierten neueren Spezifitätsgranula-Bezeichnungen synonym mit den Granula [1, 4]. Die Terminologie dieses Prozesses ist im 2. Generationen gestaffelt in Granulogenese von der neutrophilen auf die eosinophile Reihe hat zu konvergieren. Mengen gebildet. Schon 1879 hat LEUKERT [10] die Vorstellung ausgesprochen, dass sich die eosinophile Granula aus einer massenhaften Verfall der neutrophilen Granula ausserordentlich. Während LÖNNBERG [7] hiermit übereinstimmend bereits [8] *et al.* [9] ebenso wie FÄRBER und THILLY [7] bei Eosinophilen nur eine Granula-Form annehmen die sich aus Primärgranula entwickelt haben, hat ACHERMAN [12], MILLER und HILZ [13], HARTMANN und FAUPEL [1, 4] um den Nachweis einer Granulogenese über Generationen auch bei der eosinophilen Reihe bemüht. In der Tat ist es einer früheren oder späteren Granula-Form die sich im Myelozytenstadium gebildet haben [1] angeschlossen werden und.

Wenn also WARD *et al.* [24] den Begriff «Sekundärgranula» auf eine primäre Granulatausart anwenden, so muss dies zu einer nomenklatorischen Unklarheit und Irrführung Anlass geben. SM können nur dann formal als sekundäre Granulatausart aufgefasst werden, wenn sichergestellt ist, dass die letzteren kinologisch sekundäre Granulation der Eosinophilen eine einheitliche Generation darstellen. Diese Frage bedarf aber offenbar einer Klärung wie auch HILZ [12] bemerkt.

Zur Vermeidung von Missverständnissen haben wir den Begriff einer «sekundären Granulogenese» eingeführt [22]. Der Terminus «Mikrogranula» betont, dass es sich um eine Granulation kleineren kinologischen Dimensions handelt, von der bisher bekannt ist, dass kinologisch sekundäre eosinophile Granula aus Primärspezifitätsgranula [1, 4, 12] oder spezifische Granula [13] weiterentwickelt sind. Es ist jedoch der Möglichkeit, dass diese sekundären Gebilde auch aus dem kinologischen (Hilz) primären hervorkommen können, und das Mikrospezifische

fisch» ausdrücken, dass es sich um ein spezifisches zytoplasmatisches Differenzierungsprodukt reifer Eosinophiler handelt, das zumindest beim Menschen und bei Säugetieren in konstanter Homologie mit speziestypischen Varietäten vorkommt. Diese besondere Granulation stellt ein morphologisches Detail der zytoplasmatischen Organisation des Eosinophilen dar, dessen funktionelle Bedeutung noch zu klären ist. Möglicherweise handelt es sich um eine Trägerstruktur solcher Enzyme, deren Vorkommen in Eosinophilen zwar bekannt ist, deren genaue Lokalisation in Zytoplasma aber noch offen ist. So wissen wir aus biochemischen Untersuchungen von LUTZNER und BRADITT [14], dass ein erheblicher Teil der sauren Phosphatase und β -Glucuronidase nicht in den EG sondern im übrigen Zytoplasma lokalisiert ist. Zytochemisch konnten wir [23] bei Ratteneosinophilen feststellen, dass insbesondere auch die alkalische Phosphatase ausserhalb der EG gelegen ist. Ob diese und möglicherweise weitere «hämatische» Enzyme des Eosinophilen in den SM enthalten sind, wird Gegenstand weiterer Untersuchungen zur funktionellen Bedeutung dieser Körper sein.

Zusammenfassung

An 20 verschiedenen Säugetierarten aus 7 Tierordnungen wird nachgewiesen, dass reife eosinophile Granulozyten ausser den bekannten eosinophilen Granula konstant über eine weitere, bisher kaum berichtete Granulationsart verfügen die nur elektronenmikroskopisch sichtbar ist. Die Dimension solcher *spezifischen Mikrogranula* (SM) der Eosinophilen bewegt sich in einem Grossenordnungsbereich von etwa 100 nm. Die Eosinophilen des Menschen und der Säugetiere enthalten SM in verschiedenen artabhängigen Formvarietäten. Dabei handelt es sich um kugelige, hantel- oder napfförmige Körper, welche aus einem massig dichten Inhalt bestehen der von einer Einheitsmembran umgeben ist.

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Glucose-6-Phosphate Dehydrogenase Electrophoresis in Ghanaians with AA and SS Haemoglobin

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Abstract. Electrophoresis of glucose-6-phosphate dehydrogenase was carried out on 210 male Ghanaians of various ages and ethnic origins. Half of them had the normal AA haemoglobin pattern and half had sickle cell anaemia with an SS pattern. Almost half of the subjects were of Ga ethnic origin and the percentage was higher in the SS group. The overall percentage of the A type G-6-PD was 42.4. For individuals of 15 years of age or less it was 44. For individuals of Ga origin it was 46 and for the individuals with sickle cell anaemia it was 47. For individuals over 15 years of age it was 41, for non-Ga individuals it was 43 and for individuals with normal haemoglobin pattern it was 39.

Key Words: G-6-PD in Ghana; Sickle cell disease

Whereas the European usually has the B type of glucose-6-phosphate dehydrogenase (G-6-PD) the African male may have either B, A or A⁺ types. The A⁺ type has much less activity than A or B but the same electrophoretic pattern as A. Population surveys [1, 2] indicate that in Africans and persons of African descent the incidence of A and A⁺ types is approximately equal (table I). Until good evidence to the contrary is forthcoming, one may suspect that the percentage of G-6-PD deficiency in the male is approximately equal to half the percentage of the sum of A and A⁺ types.

The incidence of G-6-PD deficiency in Ghana is usually given as 22% a figure calculated from a hospital series of Akim or Ga ethnic origin [3]. A slightly higher figure, 24%, was calculated from school children of Akim origin [4] and a slightly lower figure, 20%, from newborn infants in Accra, presumably largely of Ga origin [4]. From surveys representing a much broader cross section of the population of the country, a figure of 14% was obtained [5]. Seventeen school children in the North of the

Table I G-6-PD electrophoresis (males), relationship between A and A types

Ethnic origin	Size of sample	Percent of each type			Reference
		A	A-	B	
Yoruba (Nigeria)	141	22	22	56	1
24% Negro (Brazil)	"	8	7	85	2
Negro (USA)	311	16	18	66	1

Table II Surveys for G-6-PD deficiency in Ghana (males)

Number	Ethnic origin	Age group	Deficiency %	Reference
96	Akim+Ga	children	22	3
64	Akim	children	23	4
100	mixed	adult	14	6
131	northern	adolescent	11	7
79	mostly Ga	new born	20	5
470	mixed	mixed	17	weighted average

country showed only 11% with deficiency of G-6-PD [7]. The average figure for all surveys in Ghana is 17% (table II).

In Ghana, a higher than expected incidence of G-6-PD deficiency has been reported in neonatal jaundice [5], infectious hepatitis [8, 9], in typhoid fever [10] and in sickle cell disease [11]. These findings are summarized in table III. In all of the above mentioned conditions, the high incidence of G-6-PD deficiency may be due in part to the greater likelihood of such individuals to develop jaundice and thus to come to the hospital for diagnosis [12].

The report of a higher than expected incidence of G-6-PD deficiency in sickle cell trait [11] must be regarded with suspicion because most of the AA individuals were normal volunteers while many of the AS individuals had infectious hepatitis [8]. However, in Saudi Arabia such a correlation has been proven [13].

Table III G-6-PD deficiency in different diseases in Ghana

Number	Disease	Sex	Age group	Deficiency %	Reference
270	hepatitis	male and female	mixed	40	9
36	typhoid fever	male	mixed	37	10
59	neonatal icterus	male	new-born	51	5
93	sickle cell anaemia	male	mixed	43	11
470	none	male	mixed	17	table II

The assessment of an association between sickle cell anaemia and G-6-PD deficiency is more complicated. The methaemoglobin reduction test may suggest the presence of defect when it does not exist if the blood samples are not concentrated to a haematocrit of 40% [14]. The quantitative test for enzyme activity may give normal values despite an inherited defect because of the reduced average age of the red cells [15]. For these reasons, the present study was limited to electrophoresis of G-6-PD in individuals with SS and AA haemoglobin patterns.

Materials and Methods

Blood samples were collected by venipuncture from male patients attending the sickle cell clinic and the blood was placed in acid citrate dextrose (ACD) solution and kept in a refrigerator. A control series of blood samples was taken from other male patients and volunteers. The age and ethnic origin as well as the haemoglobin pattern was recorded. It was not possible to match the two series in age or ethnic origin because most of the patients in the sickle cell clinic were young and adults of various ethnic groups. In fact the ethnic origin of the SS individuals was 40% Akan, 24% Ashanti and 36% other tribes. The ethnic origin of the AA individuals was 66% Akan, 16% Ashanti, 14% Northern and 4% other tribes.

The technique used for G-6-PD electrophoresis has been described in a WHO Technical Report [16]. However, certain modifications were made. The electrophoresis was carried out at 4°C. The density of the blood samples was 1.17. For detection 10 mg of starch was substituted for 2 mg WGE dye and the quantity of phenylhydrazine was reduced from 2 to 1 mg.

Table I G-6-PD electrophoresis (males), relationship between A and A types

Ethnic origin	Size of sample	Percent of each type			Reference
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Table III G-6-PD deficiency in different diseases in Ghana

Number	Disease	Sex	Age group	Deficiency %	Reference
200	beniolo	male and female	mixed	40	9
36	typhoid fever	male	mixed	39	10
49	neonatal icterus	male	new born	51	9
111	sickle cell anaemia	male	mixed	43	11
470	none	male	mixed	17	table II

The assessment of an association between sickle cell anaemia and G-6-PD deficiency is more complicated. The methaemoglobin reduction test may suggest the presence of defect when it does not exist if the blood samples are not concentrated to a haematocrit of 40% [14]. The quantitative test for enzyme activity may give normal values despite an inherited defect because of the reduced average age of the red cells [15]. For these reasons the present study was limited to electrophoresis of G-6-PD in individuals with SS and AS haemoglobin patterns.

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Blood samples were collected by venipuncture from male patients attending the sickle cell clinic and the blood was placed in acid citrate dextrose (ACD) solution and kept in a refrigerator. A control series of blood samples was taken from other male patients and volunteers. The age and ethnic origin as well as the haemoglobin pattern was recorded. It was not possible to match the two series in age or ethnic origin because most of the patients in the sickle cell clinic were young and individuals in the control series were of an older age and of other ethnic origin. In fact the ethnic origin of the SS individuals was 16 Ga, 11 Akan, 7 Fante, 2 Ashanti and 1 Ewe. The ethnic origin of the AS individuals was 16 Ga, 20 Akan, 10 Fante, 16 Ashanti and 1 Ewe.

The technique used for G-6-PD electrophoresis has been described in a WHO Technical Report [16]. However, certain modifications were made. The electrophoresis was carried out at 20°C. The density of the blood samples was 1.1. For the more stringent of the two methods used for 2 mg MTT dye and the electrophoresis buffer was also modified to 2.5 M Tris.

Table IV Results of G-6-PD electrophoresis (males)

Haemoglobin pattern	Age years	Ethnic origin	A type G-6-PD	B type G-6-PD
AA	15 or less	Ga	6.5 ¹	7
AA	15 or less	non Ga	14.5 ¹	23
AA	over 15	Ga	7.5 ¹	17
AA	over 15	non Ga	11.5 ¹	18
SS	15 or less	Ga	19	15
SS	15 or less	non Ga	10	19
SS	over 15	Ga	10	12
SS	over 15	non Ga	10	10

¹ One parent was Ga and one non Ga

Table V Analysis of G-6-PD type according to age, ethnic origin and haemoglobin pattern (males)

Group analyzed	A type, %	B type, %	χ^2
15 or less	44	56	0.24
Over 15	41	59	
Ga	46	54	0.8
Non Ga	40	60	
AA	38	62	1.6
SS	47	53	

Results

A total of 210 samples of blood were subjected to electrophoresis for G-6-PD (table IV). Some 42.4% showed type A while 57.6% showed type B. In table V the results are grouped according to age, ethnic origin and haemoglobin pattern, with χ^2 values for each of these classifications.

The difference in the percentages of types A and B between individuals 15 years of age or less, and individuals over 15 years of age is so small that it is more likely to be due to chance than to an actual difference.

The difference in the percentages of types A and B between individuals of Ga and of non-Ga ethnic origin is larger but below the level of statistical significance for the sample examined.

The difference in the percentages of types A and B between individuals with normal and SS haemoglobin patterns is greater, and χ^2 analysis indicates an 80 percent probability of a real difference.

Discussion

The finding of 42.4% of type A G-6-PD in a mixed Ghanaian population (predominantly of Ga ethnic origin) suggests an incidence of G-6-PD deficiency of 21% in this population. This is slightly lower than the figures given for Ga-Ga + Akim and Akim groups but is higher than the figure of 17% for Ghana as a whole and much higher than the figure of 11% for northern Ghana. These observations are compatible with the fact that in the northern part of Ghana the incidence of the S gene is about 10% while in the southern part of Ghana it is about 20% [7] and with the observation that populationwise the incidence of G-6-PD deficiency parallels the incidence of the S gene [16].

The lack of variation of G-6-PD type distribution with age is compatible with all but one study [17]. The slightly higher incidence of type A G-6-PD in the Ga as compared with the non-Ga population fits in with the fact that the incidence of G-6-PD deficiency is higher in the Ga than in the non-Ga population.

The difference in incidence of type A G-6-PD in SS individuals compared with AA individuals may be due in part to the fact that there was a higher proportion of Ga individuals in the SS group.

The fact that G-6-PD deficiency causes a greater intensity of jaundice which might lead to an increased likelihood of hospital attendance and diagnosis of sickle cell anaemia is a less plausible explanation for the higher incidence of type A G-6-PD in sickle cell anaemia.

Agreement of the present results with those most recently reported [18] and discrepancy with results previously reported by the same author in an earlier study [11] may be due in part to the fact that in the earlier study there was a somewhat more pronounced bias in favour of the Ga ethnic group in the SS population. However, the error may have been due in part to the experimental technique of the previous study, because severe anaemia is common in SS disease and blood from such individuals

may show less than normal G 6 PD activity despite the presence of the II type if the specimens are not properly concentrated [14]

There remains the possibility that the higher incidence of A type G 6 PD in sickle cell anaemia is due to a protective influence of this abnormality on the course of sickle cell anaemia [11, 18]

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Haemoglobin C in Arabs in Kuwait

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Abstract Five cases of homozygous haemoglobin C disease are described for the first time in Arabs in Kuwait. One family is Jordanian, the other is Kuwaiti. Haemoglobin C trait has also been found in other cases.

Key Words
Arabian population
Hb C disease
Hb C in Kuwait

Haemoglobin C was described by ITANO and NEEL [8]. Since then it has been reported in different races and countries: Negroes in West Africa [4], in South Africa [1], in North American Negroes [11, 13], in Lumbee Indians in North America [9], in Algerians [10], in Saudi Arabians [6], in an apparently Dutch [7], in Anglo Saxons [5] and in an Italian [3].

This are the first reported cases of homozygous haemoglobin C disease and trait in Arabs in Kuwait.

Methods

Routine haematological tests were done according to DACEY and LEWIS [2], such as haemoglobin concentration by the cyanmethaemoglobin method, haematocrit, reticulocyte count and osmotic fragility. Fetal haemoglobin was measured according to DACEY and LEWIS [2], modification of SINGER *et al* [12]. Sickling was done with *F. coli* and a known positive set up at the same time. Haemoglobin electrophoresis was done on cellulose acetate strips with barbitone buffer at pH 8.6; the strips were stained with Ponceau S and washed with 5% acetic acid. Haemoglobin A₂ was estimated by the discontinuous buffer system: this buffer pH 9.1 at the anode and barbitone buffer pH 8.6 at the cathode. The A₂ was then eluted from the strip and measured spectroscopically. Only levels above 3.5% were considered to be significant.

Table I

Case No.	Age years	Hb %	PCV %	Retic %	HbF %	HbA ₂ %	HbC %	Target cells, %	Blood film
1	2½	10.3	25	4.0	2	2.8	95	50	folded cells + hypochromia + +
2	3½	10.5	29	5.0	2	2.5	94	50	hypochromia + +
3	1½	7.4	21	2.0	2.5	2.0	95	75	hypochromia + + +
4	3	11.0	30	1.5	2	2.4	96	40	folded cells hypochromia +
5	5	10.6	28	3.0	2.5	2.8	95	60	hypochromia +

Case Reports

Case 1 The child was admitted to the hospital with jaundice. The parents stated that she has always been jaundiced since childhood, but this has increased prior to admission. History of lower limbs pain was the only significant symptom obtained. Spleen was 3.5 cm below costal margin and liver 1 cm below costal margin. The family is from Kuwait but of negro origin. The father has been married twice and the first wife is from Kuwait while the second is from Syria (mother of case 1 and 2). The family is shown in figure 1. Haematological values are shown in table I.

Case 2 Sister of case 1. She was found on family study (fig 1). No symptoms were present, however the spleen was 2 cm below costal margin and liver was not enlarged. Haematological values are shown in table I.

Case 3 This patient was admitted with chest infection. She was found to be anemic and, on routine examination in the Haematology Department, haemoglobin C was found. Spleen was just palpable and liver not enlarged. Parents are cousins and are from Jordan. The grandmother has haemoglobin C trait. The family is shown in figure 1 and haematological values are shown in table I.

Cases 4 and 5 Sisters of case 3. They were found on family study. In case 4 the spleen was 1.5 cm below costal margin and the liver was just palpable. In case 5 the spleen was 2.0 cm below costal margin and the liver was not enlarged.

Haemoglobin C trait was found on routine examination of blood samples for haemoglobin S in 3 more cases.

The osmotic fragility was decreased in all the cases of homozygous Hb C.

Comments

The incidence of haemoglobin C in Kuwait is about 0.6%. This may not be the true incidence since the disease is mild, as shown by the accidental discovery of the other homozygous cases. Certainly, the incidence is less than sickle cell anaemia or thalassemia.

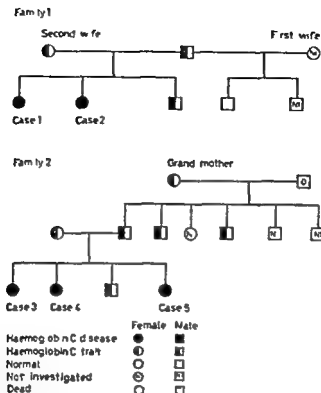


Fig 1 Pedigrees of family 1 (cases 1 and 2) and family 2 (cases 3-5)

We did not come across adult cases of homozygous haemoglobin C disease. This is probably due to the low incidence of haemoglobin C, rather than the disease causing death, as it is mild and the standard of medical care in Kuwait is reasonable, so that even cases of sickle cell anaemia are living to adulthood. Other workers reported adults with the disease, the oldest, reported by WHITEY *et al* [13], is 74 years old.

Negroid feature was noticed in the first 2 cases and this would explain that the gene may have been imported from Africa with the old slave trade in Arabia. However, the second family was Jordanian and haemoglobin C has been reported in an Italian family by DIGGS *et al* [3]. The connection between the 2 nationalities is that they are from Mediterranean origin.

We have not come across sickle cell haemoglobin C disease or thalassaemia haemoglobin C disease. This may be due to the low incidence of haemoglobin C, it has, however, been reported by other workers.

Acknowledgement We are grateful to Prof H. LEHMANN for confirming the results of our electrophoresis and for his kind help.

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Congenital Thrombocytopathy (Platelet Factor 3 Defect) with Prolonged Bleeding Time but Normal Platelet Adhesiveness and Aggregation

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Abstract A case of platelet factor 3 deficiency (thrombocytopathy) is presented. The coagulation study showed a normal plasmatic pattern but a defective prothrombin consumption. The thromboplastin generation was defective too and was corrected by the substitution of the patient's platelets with normal platelets or with a platelet substitute. The platelet factor 3 availability was abnormal. On the contrary, platelet adhesiveness to glass and ADP, adrenaline or collagen induced platelet aggregation were normal. The parents and a sister of our propositus presented a normal coagulation pattern.

Key Words

Bleeding disorders
Blood coagulation
Platelet factor 3 deficiency
Thrombocytopathy

Bleeding disorders with prolonged bleeding time and no concomitant plasmatic defect still represent a very complicated matter [3, 4, 8, 14, 15, 21, 23]. The recent studies seem to have confused the pattern so that any classification attempt is a very difficult task. The simplest classification is the one that takes into account fundamental platelet functions and platelet number, namely clot retraction, platelet factor 3 production, platelet count. Taking into account these considerations, 3 major congenital platelet syndromes may be indicated, namely thromboasthenia, thrombocytopathy and thrombocytopenia. This classification is obviously approximate but it is still clinically satisfactory.

Recently several mixed or 'new' syndromes have been described [13, 16-18, 20]. These conditions still await final classification and may be considered, at least temporarily, as variants of one of the main conditions. This appears fully justified if one takes into due account the fact that only in recent years a satisfactory platelet function tests battery has become available. An exception may be postulated for the 'dystrophic thrombocy-

taire' and for the 'Portsmouth syndrome' which seem to have been satisfactorily established [1, 5, 6, 11, 25]

The object of the present paper is to present a patient with a prolonged bleeding time and an isolated platelet factor 3 deficiency, namely a case of thrombopathy or thrombocytopathy. We think, in fact, with QUICK and HUSSEY [17] and QUICK [18] that this name should be limited to conditions showing a defective platelet factor 3 activity

Case Report

The proband is an 11 year-old boy who was first studied by us in October 1968 and was followed ever since as an outpatient. Parents were not consanguineous and were asymptomatic. Family history was negative for bleeding disorder but for the fact that the paternal grandmother died at the age of 33 because of massive *post partum* hemorrhage.

The child was born at term and no abnormality was noted in the neonatal period. No undue bleeding was noted at the separation of the umbilical cord. At the age of 2, the patient started complaining of epistaxis. Easy bruising was first noted at approximately the same time. At the age of 7 the patient presented spontaneous bleeding from the right tonsil during an acute tonsillitis episode. The bleeding was so marked as to require admission to a local hospital where cauterization of the bleeding surface was carried out. About 2 months later, the patient presented again bleeding from the right tonsil and had to be admitted again to the local hospital. The patient was transfused with 250 ml of fresh whole blood and transferred to the Pediatric Department of our University. On the admission, the patient was not bleeding. A preliminary coagulation study showed no plasmasmic abnormalities but a prolonged bleeding time and a defective prothrombin consumption. Further studies confirmed the prothrombin consumption defect and led to the diagnosis of thrombocytopathy. During admission, the patient presented 3 additional episodes of bleeding from the pharynx which promptly subsided upon transfusion of platelet rich plasma.

During the past 3 years, the patient has been followed by us as an outpatient and no further bleeding episode occurred. Occasional epistaxis and easy bruising, however, were present.

Material and Methods

Material and methods have been discussed in detail elsewhere [9-10]. Only new data will be given herein.

Platelet rich plasma was obtained by centrifuging non-contacted citrated plasma at 1000 g for 10 min.

Prothrombin consumption after addition of normal platelets was carried out 2 h after the standing of the blood at 37°C. Dried thrombocytes as supplied by Behringwerke Laboratories, Marburg FRG were used.

Thromboplastin generation test was carried out according to the Oxford method [2]. Patient's platelets or normal platelets were resuspended to $1/10$ of the original plasma volume, stirred with a glass rod for 5 min and stored at -20°C for a few days before use. Aliquots were frozen and thawed only once.

Platelet factor 3 availability test was carried out according to the method proposed by SPAET and CRYSTON [22]. Kaolin NF CB443 as supplied by Matheson Coleman & Bell, New York was used. The Russell viper venom was used in a 1:100,000 dilution in Michaelis buffer.

Platelet adhesiveness to glass was carried out according to a modification of HELLEM's method [12]. The glass beads used were 1 mm in diameter and the height of the column was 10 cm whereas the filtration time was 30 sec. Platelet adhesiveness was measured also according to SALZMAN's method [19]. In this latter case the material used was supplied by Becton & Dickinson Laboratories, Rutherford N.J., USA.

Platelet aggregation was carried out according to the method of COOK and SYMONS [7] using an aggregator supplied by Evans Electroselenium Ltd., Halstead Essex, England. The reagents supplied by Stago Laboratories, Asnières, France were used. Adrenaline was added in the amount of $1\text{ }\mu\text{g/ml}$ of plasma or $0.5\text{ }\mu\text{g/ml}$ of plasma. Collagen was added in the amount of $40\text{ }\mu\text{g/ml}$ of plasma. The collagen solution was prepared by reconstituting the Stago reagent with 0.1 N acetic acid. The solution was then kept at 4°C for at least 36 h. Just before use the solution was diluted 1:10 with Michaelis buffer pH 7.3 and incubated at 33°C for 3–7 min.

Results

The coagulation study is summarized in table I and in figure 1. The plasmatic coagulation system was normal and no hyperfibrinolysis was present. Bleeding time was prolonged whereas clot retraction was normal. Thromboplastin generation was abnormal when the patient's platelets were used in the generation mixture. The substitution of the patient's platelets with normal platelets or with a platelet substitute, corrected the abnormality (fig. 1). The Stypven clotting time was moderately prolonged and the platelet factor 3 availability test was defective. Prothrombin consumption and thromboplastin generation were defective and were corrected by the addition of normal platelets or of a platelet substitute. ADP, adrenaline and collagen-induced platelet aggregation was normal. Platelet adhesion to glass was normal with both the methods used. The administration of platelet-rich plasma corrected the prolonged bleeding time whereas the administration of platelet-poor plasma had no effect (table II).

The parents and the sister of our propositus were all found to have a normal coagulation pattern (table III).

Table 1 Coagulation study

Test	Propositus	Normal values	Comments
Blood platelets, $\times 10^9/\text{mm}^3$	180	150-350	normal morphology
Clot retraction	complete in 7 h	complete in 12 h	
Bleeding time (Duke), min	17-30	3-5	extremes of several determinations
Bleeding time (Ivy), min	11	4-6	
Glass clotting time, min	7	5-9	
Prothrombin consumption, %	40	>90	
Prothrombin consumption after the addition of normal platelets, %	>90	>90	
Serum prothrombin time, sec	18.9	>22	
Serum prothrombin time after the addition of normal platelets, sec	26.6	>22	
TGT	28 sec in 6 min	<15 sec in 6 or 8 min	
TGT + normal platelets	15 sec in 6 min	<15 sec in 6 or 8 min	
TTP, sec	41.5	35-45	
Recalcification time, sec	180	100-180	
Prothrombin time, sec	13.5	13-14	
Soybean clotting time, sec	29.6	15-20	
Platelet factor 3 availability, sec	40	19	clotting times observed after 30 min incubation
Fibrinogen, mg %	400	250-450	
Factors II, V, VII, IX, X, XI, XII	normal		
Thrombelastogram			
a	13	10-20	
k	6	6-12	
am	60	50-66	
Thrombin time, sec	19	18-25	
Platelet adhesiveness (Hiss and Rodin, %)	20	10-40	
Platelet adhesiveness (Sakuma), %	37	20-50	
Platelet aggregation ADP (5 μg)	6	6	maximal amplitude (optical density variations) in conventional test
ADP (0.5 μg)	37	47	
Adrenaline	5	6	
Collagen	6	6	

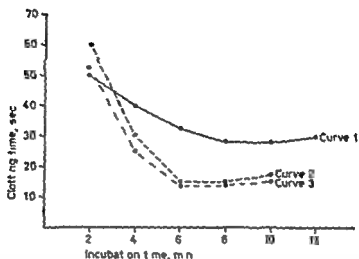


Fig 1 Thromboplastin generation test Curve 1 incubation mixture containing patient's serum, patient's adsorbed plasma and patient's platelets Curve 2 mixture containing patient's serum, patient's adsorbed plasma and normal platelets Curve 3 mixture containing patient's serum, patient's adsorbed plasma and a non activated cephalin preparation (Platelin) CaCl_2 was present in equal amounts in each generation mixture

Table II Bleeding time (Duke) after plasma transfusion

	Bleeding time min	Comment
Basal value	>30, left ear	date october 25, 1968
2 h after the infusion of platelet poor plasma (260 ml)	17, right ear	no decrease in bleeding from the pharynx was noted after this trans- fusion
24 h after the same transfusion	22, left ear	
Basal value	>30 right ear	date november 16 1968
2 h after the transfusion of platelet rich plasma (200 ml)	7, left ear	a decrease in bleeding was noted after this transfusion
24 h after the same transfusion	>30, right ear	

Table III Coagulation data in the patient's relatives

Relative	Bleeding time min	Serum prothrombin time sec	Prothrombin consumption %	Platelet count $\times 10^3 \text{ mm}^3$	Clot retraction	PT sec	PT sec
Father	3.5	29	>90	230	+	39	14
Mother	4	30	>90	200	+	38	13
Sister	4	28	>90	300	+	40	14
Normal values	2-5	>22	>90	150-350	+	35-45	13-14

Discussion

The main criteria for the diagnosis of platelet factor 3 defect or thrombocytopathy are normal platelet count, prolonged bleeding time, defective prothrombin consumption and normal plasmatic coagulation factors. Our patient fully meets these criteria. The possibility of an acquired condition may be ruled out because of the lifelong bleeding tendency, the lack of any associated disease and the constancy of our coagulation findings over a 4 year period. The possibility that our patient was a case of Portsmouth syndrome may be ruled out because of the normal reactivity of the platelets to adrenalin and collagen. Furthermore, in this latter syndrome the platelet factor 3 defect does not seem to be constant [6, 11, 24]. The normal platelet morphology observed in our patient, rules out the possibility of a case of 'dystrophic thrombocytaemia'. The bleeding manifestations are those seen in patients with thrombocytopathy. The prolonged bleeding time has to be ascribed only to the platelet factor 3 defect since no defect was noted in the platelet adhesiveness and aggregation or in the plasmatic coagulation pattern. This indicates that in our proposition the platelets were able to adhere normally to the wound edges but a defective plug formed because of the lack of platelet factor 3. The possibility of a concomitant vascular defect, however, cannot be ruled out.

ADP release from platelets in our case was not measured but there is indirect evidence that it was normal. In fact, glass adhesion and platelet aggregation to collagen, which are phenomena known to require ADP release were normal [24].

It was generally believed that 2 phenomena were associated [24] Our findings suggest that these 2 platelet activities may be, in certain conditions, independent. In other words, a defective factor 3 production or release may be noted even though a normal ADP platelet release is present.

The almost complete correction of the bleeding time after transfusion of platelet rich plasma is in agreement with our diagnosis too. In this regard, it is interesting to note that 24 h after the transfusion the bleeding time was again severely prolonged. This behavior is compatible with the known short survival of homologous human platelets.

It has been postulated that thrombocytopathies could be divided in 2 subtypes: real defect in production and defect in release. We think this is irrelevant for clinical purposes. Furthermore, the techniques used to free factor 3 from platelets such as sonification seem approximate at best [15]. However, the results of platelet factor 3 availability test obtained in our case, strongly indicate a real deficiency rather than a defective release.

As far as heredity is concerned we may only state that the normal coagulation pattern found in the propositus' father, mother and sister seems consistent with an autosomal recessive type of inheritance. No other relatives of our propositus were available for study.

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Hereditary Persistence of Fetal Hemoglobin and β -Thalassemia in a Turkish Child

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Abstract A Turkish boy with hereditary persistence of fetal hemoglobin β thalassemia combination is described. His Hb F was very much elevated and no Hb A could be shown in his hemolyzate. His Hb A₂ was low normal corresponding to the Negro counterpart. His father had β thalassemia trait with elevated Hb A₂ and his mother was heterozygote for high persistence fetal hemoglobin with the Hb F and Hb A₂ values resembling reported Greek AF cases.

Key Words
Hemoglobin F persistence
Hemoglobinopathies
Thalassemia

Hereditary persistence of fetal hemoglobin (HPFH) was first described by EDINGTON and LEHMANN in 1955 [1]. It has recently received increased attention since the heterogeneity of fetal hemoglobin in homozygotes and in β thalassemia-HPFH heterozygotes has been shown [2, 3]. Most of the reported cases of HPFH [2, 3] have been in Negroes [3, 4] and Greeks [3-5] but cases among Italians [6], Sicilians [7], Portuguese-Indians [8], Puerto Ricans [9] and Thais [10] are also recorded.

In homozygous HPFH cases, only Hb F is demonstrable in the red cell hemolysate, and no trace of Hb A or A₂ is present. The heterozygotes have 10-38% of Hb F, which is more or less evenly distributed in the erythrocytes, with usually decreased Hb A₂ concentration. Heterozygotes for HPFH β thalassemia have an average of 72% of Hb F with a normal or elevated Hb A₂ [4].

Although β thalassemia is relatively common in Turkey [11] the combination of this disease with HPFH has not been reported. In fact, this is

the first report from this country of HPFH cases with or without the thalassemia combination

Case History

H S (HCT 126179), a 6-year-old Turkish boy, was seen at Hacettepe Children's Hospital Medical Center because of pallor and anorexia. Paleiness was first noticed at about 2 years of age and he had jaundice, for a short duration 2 years prior being seen in this hospital.

On examination, the child looked pale and subicteric. A grade 2/6 systolic murmur was best heard over precordial area which was not transmitted. The spleen was not palpable but the liver extended 1.5 cm below the right costal margin. Other clinical findings were noncontributory.

Laboratory results: Hb 9 g/100 ml, Hct 27%, WBC 14,500/ml and a reticulocyte count 0.0%. On peripheral smear mild hypochromia, microcytosis, anisocytosis and few target cells were seen. With the exception of slight elevation of serum bilirubin (total 2.3 mg%, unconjugated 1.4 mg%), other liver function tests were within normal limits.

The patient's fetal hemoglobin (Hb F) and Hb A₂ concentrations were 82–85 and 1.75%, respectively, and no Hb A was shown by starch gel (pH 8.6) and agar gel (pH 6.45) electrophoresis. Erythrocyte osmotic fragility was markedly decreased. Inclusion bodies were not seen after staining with brilliant cresyl blue. The concentration of Hb F in red cell hemolysates of mother and father were 31.5 and 3.3%, respectively. Their Hb A₂ values were 2.42 and 5.8% in the same order. No abnormal hemoglobin could be shown in the mother's or in the father's hemolysates by starch gel and agar gel electrophoresis. However, elevated Hb F in the mother's and Hb A₂ in the father's hemolysate was readily detected by agar and starch gel electrophoresis, respectively. The mother's red cell morphology showed no abnormality and inclusion bodies with brilliant cresyl blue were not present. The osmotic fragility of red cells from both parents was slightly decreased. Staining of the peripheral smears for fetal hemoglobin [method of KALMUTTA and BETTER (13)] revealed this hemoglobin to be uniformly distributed in all red cells of the child and his mother and only in occasional red cells of father.

Methods

Hemograms were obtained by standard techniques. The Hb concentration was determined by the cyanmethemoglobin method, and the microhematocrit was used for measuring packed cell volume. Intracythrocytic iron (total) and fetal hemoglobin assays were made as described by DACEY and LEWIS (12) and KALMUTTA and BETTER (13). Starch gel and agar gel electrophoresis were performed according to the method of SWENDE (14) and ROBINSON *et al.* (15), respectively. In unaltered osmotic fragility (16), and Hb A₂ concentrations were determined according to DACEY and LEWIS (12), SPECTER *et al.* (16) and HUGHES and DACEY (17), respectively.

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Ultrastructural Features of Phytohemagglutinin and Concanavalin A - Responsive Lymphocytes in Chronic Lymphocytic Leukemia

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Abstract. Peripheral blood lymphocytes from patients with chronic lymphocytic leukemia (CLL) had diminished and/or delayed *in vitro* responses to both phytohemagglutinin (PHA) and concanavalin A (Con A) as determined by thymidine incorporation in ³H DNA over a 9-day incubation period. The cytoarchitectural features of CLL lymphocytes stimulated by either of the mitogens were similar to those observed in transformed normal cells. However, as shown by planimetric measurements, the mean cell area and the nuclear, cytoplasmic, and mitochondrial areas were diminished in comparison to PHA stimulated normal cells. The data suggest that some CLL lymphocytes which transform with PHA or Con A are residual normal thymus-dependent (T) lymphocytes, and others may be derived from the metabolically defective leukemia cell population.

Key Words:
Concanavalin A
Electron microscopy
Lymphocytic leukemia
Lymphocyte transformation
PHA

Incubation with phytohemagglutinin (PHA) or concanavalin A (Con A) *in vitro* induces transformation of normal human peripheral blood

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lymphocytes, and maximum DNA synthesis occurs at day 3 of the culture period [13]. The fundamental mechanisms underlying lymphocyte activation are yet to be determined, nevertheless, binding of the mitogen to the plasma membrane [20, 24, 27] appears to be an important requisite for the initiation of this process [15].

Lymphocytes from patients with chronic lymphocytic leukemia (CLL) have a reduced number of plasma membrane receptor sites for PHA [23] and Con A [26] and show a diminished and/or delayed proliferative response to various phyto mitogens *in vitro* [1, 5, 17, 21, 22, 32]. The impaired response may be the consequence of stimulation either of residual normal cells and/or of stimulation of defective leukemic cells. In order to further investigate the nature of the mitogen responsive cell population in CLL, we have incubated blood lymphocytes from normal individuals and CLL patients with PHA or Con A *in vitro* and have compared the fine structural features of transformed normal and CLL lymphocytes.

Materials and Methods

Nylon column purified peripheral blood lymphocytes from 8 healthy individuals and 13 patients with CLL (lymphocyte counts ranging from 12,400 to 49,800 μ l), were cultured for 3, 5, 7 and 9 days in the presence of PHA-P (Difco Lab., Detroit, Mich., USA) in Eagle's Minimum Essential Medium, Spinner-Modification (MEM-S, Grand Island Biol. Co., Grand Island, N. Y., USA) containing 20% fetal calf serum, 0.002 mmoles L-glutamine, 100 IU penicillin, and 100 μ g streptomycin/ml. Cells from 4 of the normals and 2 of the CLL patients were stimulated with Con A (Difco, Lab., Sigma Chemical Co., St. Louis, Mo., USA). Optimal doses of the mitogens were determined by dose response curves. Details of the culture procedure have been reported [6].

Four hours before harvest, the cultures received 20 μ Ci 3 H thymidine (specific activity 20 Ci/mmole, New England Nuclear Corp., Boston, Mass., USA), and 3 H-thymidine incorporation into DNA was determined [25].

For electron microscopy, the cells were fixed in 1.5% redistilled glutaraldehyde, washed in 0.05 M sodium cacodylate, and postfixed in Millonig's phosphate buffered osmium tetroxide. The pellets were dehydrated through serial changes of ethanol followed by propylene oxide and embedded in Epon 812. Thin sections were prepared on an LKB Ultratome III, and doubly stained with 1% uranyl acetate and lead citrate. The material was examined in a Siemens 101 electron microscope at primary magnifications from 3,000 to 40,000.

Planimetry was performed on micrographs of cells sectioned through comparable planes for resting (normal $n = 89$, CLL $n = 110$) and PHA activated lymphocytes (normal $n = 47$, CLL $n = 55$).

Table I ^3H thymidine incorporation into DNA by normal and CLL lymphocytes incubated *in vitro* with PHA over 9 days (cpm/culture, values of unstimulated control cultures subtracted)

	Peripheral lymphocyte count μl	3-day response cpm	Maximal response cpm	Day
<i>CLL patients</i>				
1 Sch	47,900	35	249	5
2 Bl	45,900	266	452	7
3 Ma	23,600	275	1,828	5
4 Hs	45,400	517	3,194	5
5 Wi	25,500	1,059	4,693	5
6 Se	14,400	3,554	8,740	5
7 We	31,300	1,140	14,864	7
8 Le	44,200	8,544	16,914	9
9 B	12,400	-	23,144	5
10 Re	46,900	5,619	28,615	5
11 Li	32,000	-	30,514	5
12 Ir	41,900	1,416	42,649	9
13 Pe	20,300	25,656	47,721	5
<i>Normals (n=8)</i>				
Mean		57,747	57,747	3
SEM		8,171	8,171	

Table II ^3H thymidine incorporation into DNA of normal and CLL lymphocytes incubated *in vitro* with Con A over 9 days (cpm/culture, values of unstimulated control cultures subtracted)

	Peripheral lymphocyte count μl	3-day response cpm	Maximal response cpm	Day
<i>CLL patients</i>				
12 Ir	41,900	923	923	3
13 Pe	20,300	8,211	19,913	5
<i>Normals (n=8)</i>				
Mean		67,607	67,607	3
SEM		4,104	4,104	

Table III Cell nuclear, cytoplasmic, and mitochondrial area and number of mitochondria in comparable cell sections of normal and CLL lymphocytes during a 7-day incubation with PHA

		Unstimulated		Activated by PHA					
		mean	SEM	day 3		day 5		day 7	
				mean	SEM	mean	SEM	mean	SEM
Sectional area μ^2									
Total cell	normal	24.19	4.54	134.29	10.06	117.25	4.87	64.56	4.59
	CLL	20.37	3.92	67.50	6.35	79.81	7.02		
Nucleus	normal	13.31	3.14	62.86	3.85	48.85	2.58		
	CLL	13.03	3.01	29.72	3.07	28.52	2.34	26.09	3.47
Cytoplasm	normal	10.88	3.20	71.43	7.64	68.97	4.05		
	CLL	7.33	2.72	38.78	4.86	51.29	5.19	38.47	3.19
Mitochondria	normal	1.06	0.80	7.72	0.97	7.66	1.19		
	CLL	1.37	1.10	5.32	1.04	4.51	0.68	4.53	0.94
Number of mitochondria/cell section									
	normal	4.92	3.49	8.55	1.12	10.92	1.06		
	CLL	5.41	4.53	10.24	1.54	9.04	1.09	14.58	3.63

Results

As shown in tables I and II, lymphocytes from the CLL patients revealed a diminished and/or delayed ^3H thymidine incorporation into DNA after incubation with either PHA or Con A in comparison to normal lymphocytes. The magnitude and time course of the response had no relationship to the peripheral lymphocyte counts.

The results of the planimetric measurements are presented in table III. Unstimulated CLL cells showed only a slightly diminished mean cell area, due to a decreased cytoplasmic content. After stimulation with PHA, there was an increase in the total cell area, as well as in the nuclear, cytoplasmic, and mitochondrial area and in the number of mitochondria in both normal and CLL lymphocytes. The mean nuclear and cytoplasmic size, however, were considerably smaller in PHA stimulated CLL lymphocytes than in normal transformed cells, resulting in a decreased mean total cell area. Although the number of mitochondria per cell profile was

similar in both transformed normal and CLL cells, their total area was diminished in CLL cells.

The ultrastructural changes in normal human lymphocytes during PHA stimulation include enlargement of the nucleus with abundant euchromatin and prominent nucleoli, well-developed Golgi zones, many lysosomes, and increased number and configurational changes of mitochondria, ribosomal aggregation, and some strands of rough-surfaced endoplasmic reticulum [3, 8, 9, 11, 35]. In general, transformed CLL lymphocytes showed similar morphologic alterations (fig. 1) however, the number of lysosomes was diminished in comparison to transformed normal lymphocytes (fig. 2). In some cells, irregularly shaped nuclei, lipid vacuoles, and endocytic vacuoles were prominent (fig. 3). Many transformed CLL lymphocytes were smaller (intermediate-sized) than corresponding normal cells.

The general cytoarchitectural features of normal lymphocytes transformed with Con A were similar to those present in PHA-stimulated cells. Ribosomal aggregation was prominent in Con A-stimulated lymphocytes (fig. 7) and many cells showed several strands of electron-dense endoplasmic reticulum with rare attached ribosomes (fig. 5, 7, 8). Some mitochondria were vacuolated and contained areas with loss of cristae (fig. 5, 7, 8). Endocytic vacuoles which contained finely granular material were present in some sections. This material was also present in proximity to cell membranes and probably consisted of glycoproteins from the culture medium which were precipitated by Con A [16] (fig. 5). In the Golgi region microtubules and microfilaments were discernable (fig. 7). Rarely, Con A transformed normal lymphocytes showed uropod formation (fig. 6). The uropods were characterized by the presence of the Golgi complex, many lysosome-like organelles, mitochondria, and lipid bodies (fig. 6). As in PHA cultures, less than 20-30% of CLL lymphocytes were transformed in cultures incubated with Con A for 3 days. Extensive cell agglutination was present. At 3 and 5 days of incubation, many intermediate-sized blastoid cells were observed. These cells showed only slight increase in cytoplasmic area and contained polyribosomes, few strands of rough surfaced endoplasmic reticulum, several small swollen mitochondria, and a relatively small Golgi complex. The large nucleus contained mainly euchromatin with some clumps of heterochromatin and prominent nucleoli. Con A transformed CLL cells showed abundant ribosomal aggregation (fig. 4) and in some cells formation of large endocytic vacuoles was observed (fig. 4).



Fig 1 Cells from 3 day culture of C11 lymphocytes incubated with PHA. The large blast like cell contains a euchromatic nucleus, prominent nucleolus (N), many mitochondria (m), strands of rough surfaced endoplasmic reticulum (RER, arrows) and a few granules (G). Several intermediate sized transformed cells (t) and small lymphocytes (l) are present. $\times 5,600$.

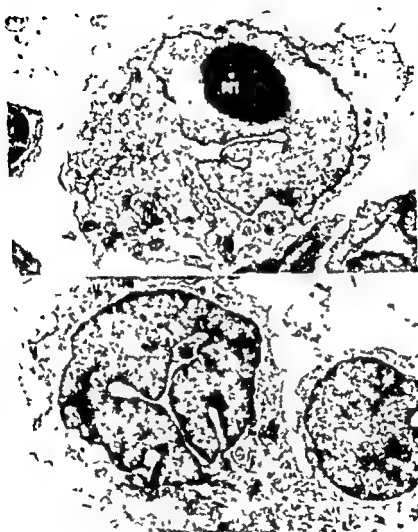
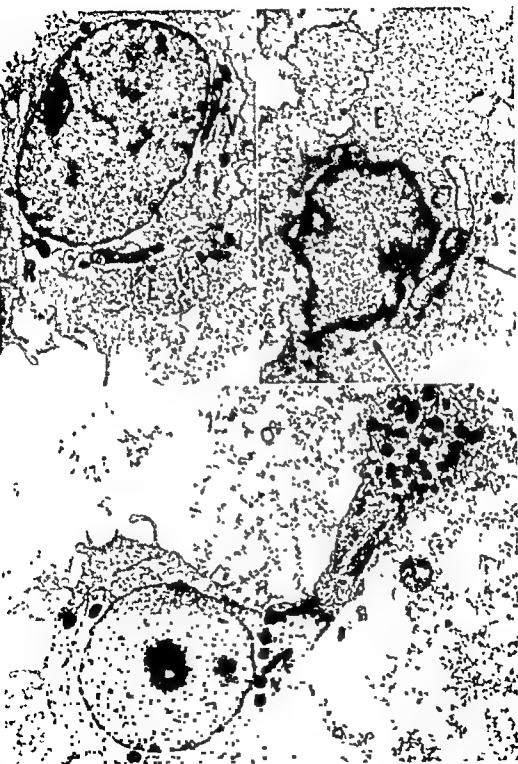


Fig. 2. Transformed cell line 7 day culture of CLL lymphocytes incubated with PHA. The large nucleus contains many nucleonemata (NL). The cytoplasm has many mitochondria (m), strands of RER and a well-developed Golgi zone (G). Cytoplasmic granules are not evident in this section ($\times 8000$).

Fig. 3. PHA transformed CLL cell in 11 day culture. Nuclear bodies (NB) represent lipid droplets (NL) and a large electron-dense nucleus (N) are evident ($\times 8000$).



Discussion

The fine structural features of normal lymphocytes transformed with Con A have previously been briefly reported [8, 9, 12]. The sequence of morphologic events resembles that which occurs with PHA stimulation, and the spectrum of transformed cells observed is similar to that present in PHA stimulated cultures [3, 8, 9, 11, 35]. The possibility that the alterations in rough-surfaced endoplasmic reticulum and mitochondria in Con A stimulated cells are related to indirect effects of the mitogen on the cells cannot be excluded.

'Resting' blood lymphocytes from patients with CLL have been shown to differ from unstimulated normal cells in their slightly decreased mean total cell area due to reduced cytoplasmic content [30-31], and a diminished number of lysosomes per cell profile [10]. In the only previous electron microscopic study of PHA transformed CLL lymphocytes, CLAUSEN and BOLANDER [7] reported observations based on examination of 8 lymphocyte cultures obtained from one normal control and 3 patients with CLL. Although these investigators did not quantitatively assess cell or organelle size, they noted an intermediate-sized blastoid cell type and some large blastoid cells which showed no significant morphologic differences from transformed normal cells [7]. In the present study, we have demonstrated that after stimulation with PHA *in vitro*, the area of the total cell nucleus and cytoplasm of many transformed CLL lymphocytes is significantly reduced in comparison to stimulated normal cells. In general, the cytoarchitectural features of the transformed cells in CLL lymphocyte cultures are similar to those of transformed normal cells except for a reduced number of lysosomes [CORREY *et al.*, in preparation]. Furthermore, incubation of CLL lymphocytes with Con A results in transformed cells which resemble those which occur with PHA.

The majority of blood lymphocytes from most CLL patients has easily detectable surface bound immunoglobulin [for references, see 2] consid-

Fig. 4. Con A transformed CLL cell in 4-day culture. Many ribosomal aggregates (R), pin vacuoles (V), and endocytic vacuoles (E) are present. $\times 9,800$.

Fig. 5. Con A transformed normal lymphocyte in 3-day culture. An endocytic vacuole (E), strands of ER with few attached ribosomes (arrows), and extracellular space (arrowhead) can be seen. $\times 8,400$.

Fig. 6. Transformed cell in 5 post-conc steped from 3-day culture of normal lymphocytes incubated in Con A. $\times 9,000$.



Fig 7 Con A transformed normal lymphocyte in 3-day culture. In the large Golgi zone, there are microtubules (T). The ribosomes are aggregated (arrows) and many strands of endoplasmic reticulum with few attached ribosomes are present $\times 400$

Fig 8 Cell in 5-day culture of normal lymphocytes incubated with Con A. Several mitochondria show areas devoid of cristae (arrows) $\times 9600$

ered to be a characteristic property of bone marrow-dependent (B) lymphocytes. Evidence from animal experiments suggests that PHA selectively activates thymus-dependent (T) lymphocytes [18]. Similarly, in mice, Con A induces proliferation of unselected T cells [19] and lymphocyte responsiveness to Con A is abolished by complete removal of T cells [33]. No significant differences were observed, however, in the average density of binding sites for Con A on T versus B lymphocytes [14, 33].

If CLL cells belong to the B cell population, then the impaired *in vitro* response to both PHA and Con A might be explained by a decreased number of normally reacting T lymphocytes. A delayed response to PHA has been reported for cultures with reduced concentrations of normal human lymphocytes [28]. The reduced reactivity of CLL lymphocytes, however, could also be related to the decreased number of membrane receptor sites for PHA [23] and Con A [26]. Since some transformed CLL cells are morphologically indistinguishable from transformed normal cells, it is likely that at least some residual normal T cells are stimulated in PHA and Con A cultures of CLL lymphocytes. Very recently [34], a mitogen responsive T cell population has been isolated from peripheral blood of CLL patients. This finding [34] is evidence that a normal population of T lymphocytes is present in CLL, which is diluted by leukemic cells which have some properties of B cells [2].

Furthermore, as in cultures of PHA stimulated normal human lymphocytes [4], uropod bearing cells were observed in mitogen-stimulated CLL lymphocyte cultures (fig. 6). In guinea pigs uropod bearing cells lack surface immunoglobulins [29] and the uropod is considered to be a characteristic of T cells. Many transformed CLL cells, however, differed from PHA or Con A transformed normal cells in nuclear and cytoplasmic size and in the number of lysosomes. These observations suggest that some transformed CLL cells probably are abnormal cells. Since various metabolic and functional abnormalities have been reported in unstimulated and stimulated lymphocytes from patients with CLL, the diminished and delayed response of CLL cells to phytoantigens appears to be in part the consequence of activation of defective leukemic cells. However, at present the possibility cannot be excluded that the intermediate sized cells represent incompletely transformed normal cells.

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Diisopropylfluorophosphate Uptake by Granulocytopoietic Cells in Chronic Myeloid Leukaemia and in Normal Individuals

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Abstract Bone marrow cells from normal and leukaemic individuals were incubated with tritiated diisopropylfluorophosphate (DIF). Stained cells were classified cytologically and photographed. Following autoradiography the grains overlying the same cells were counted. Myeloblasts took up extremely little DIF. Promyelocytes and, to a slightly lesser extent, myelocytes incorporated DIF very actively. A lower but rather uniform activity was observed in the more mature forms. The grain count varied considerably between individual cells within each maturation compartment.

Key Words
Autoradiography
Diisopropylfluorophosphate
Granulocytopoietic cells
Myeloid leukaemia

Radioactive diisopropylfluorophosphate (DIF) has been used to label granulocytes in kinetic studies, either by intravenous infusion of DIF or *in vitro* followed by reinfusion of the labelled blood [1, 2, 4-7]. From the disappearance rates of labelled cells the intravascular granulocyte half life has been deduced.

The uptake of DIF-³H by granulocyte precursors and mature cells has been investigated in an earlier study by KERN *et al.* [8]. It was concluded that myeloblasts showed very little uptake, myelocytes a high uptake and the other cell types intermediate relative labelling. However, due to technical factors, promyelocytes and myelocytes could not be clearly distinguished.

In the present work, the DIF-³H uptake was determined in cells belonging to the various granulopoietic differentiation and maturation compartments, using a two-step method which overcame the old "scully"

Materials and Methods

Three healthy volunteers were studied (No 1 2 and 3) None of them showed signs of active infection or had any history of recent infections Three Philadelphia chromosome positive patients with chronic myelogenous leukaemia (CML) in the chronic phase were selected (No 4 5 and 6) Their peripheral leucocyte counts were 6 000 12 400 and 23 900 μ l respectively Differential counts of the peripheral blood in the three cases showed 9 0 and 9% of immature granulocyte precursors The haemoglobin concentrations ranged between 12.5 and 15.4 g/100 ml the platelet counts between 356 000 and 686 000/l Patient No 6 was treated with busulphan 2 mg each day The other two patients did not receive therapy at the time of investigation

Bone marrow cells were obtained by sternal puncture The marrow was suspended in 10 ml of TC 199 medium containing 25 μ Ci DFP 3 H (The Radiochemical Centre Amersham England specific activity 3.3 Ci/mmol) and 100 IU of heparin Incubated for 45 min at 37°C and agitated intermittently to counteract sedimentation The cells were then washed twice in AB serum the cells being spun down by centrifugation at 150 g The final cell button was suspended in a volume of AB serum only slightly larger than the volume of the cells Films of this suspension were prepared on cleaned slides and quickly dried with a household hairdryer, fixed for 5 min in methanol and stained with May Grünwald Giemsa

Areas with well spread cells were photographed on Polaroid® films in a Leitz Orthoplan microscope using the 100 \times oil immersion objective The cells were classified cytologically and their positions on the moving stage of the microscope were noted In each patient from 27 to 110 cells (mean 49) of each morphological type were photographed

As it was intended to correlate morphological character and DFP uptake in the cells, the next step was to prepare autoradiographs of the smears However this posed a technical problem When slides are dipped into the liquid emulsion the adhering film tends to be thicker towards the distal end of the slide The mean thickness of our films is about 2 μ m as found by direct measurements and we have calculated the mean range of the β -emission from tritium to be 2.5 μ m in the film using an empirical equation found by LEVI [9] which shows us that sensitivity of the emulsion varies along its length As a consequence of this we have only photographed cells in an approximately 5 mm wide band across the middle of each slide

After removal of the immersion oil in 4 changes of xylol the slides were destained in methanol containing 0.5 ml of concentrated hydrochloric acid per litre (3 changes) and finally rinsed in pure methanol After drying in air the slides were immersed in a solution containing 100 mg of gelatine and 20 mg of chromealum in 200 ml of distilled water and left to dry and harden overnight in a dust free atmosphere This greatly facilitated the adherence to the slides of the Ilford K2 liquid nuclear emulsion The emulsion was diluted 1:1 in distilled water held at 42°C in a water bath The slides were dipped once left to gel on the table and dried at approximately 40°C on a hotplate The drying time was about 10 min after which the slides were stored in boxes with silica gel at 4°C and exposed for about 24 h

Table 1 The mean grain counts found in DFP labelled cells following autoradiography

Case No.	Healthy controls			Promyelocytes			Myelocytes			Metamyelocytes			Stab forms			Segmented		
	mean	n	range	mean	n	SD	mean	n	SD	mean	n	SD	mean	n	SD	mean	n	SD
	grain count			grain count			grain count			grain count			grain count			grain count		
1	1.61	31	0-5	48.9	32	37.3	26.9	44	11.7	19.3	37	7.1	19.9	40	5.3	17.9	39	5.6
2	4.01	34	0-40	33.3	34	23.2	29.8	48	13.1	15.7	38	6.1	16.4	76	5.7	14.8	93	5.3
3	6.41	49	0-34	38.1	47	17.0	32.5	110	12.5	24.8	46	8.5	26.3	35	9.1	32.0	110	11.6
4	0.94	37	0-3	24.7	36	12.5	18.0	51	7.1	13.2	36	3.2	13.4	43	3.3	12.8	56	3.2
5	2.31	33	0-24	18.2	35	19.0	32.0	67	14.6	18.8	27	7.0	23.0	30	9.4	19.4	42	6.8
6	1.25	16	0-19	29.0	36	13.5	23.4	84	9.9	13.1	40	5.7	13.6	45	4.8	12.9	42	5.1

(Cases 1-3) = healthy volunteers cases No. 4-6 = patients with chronic myelogenous leukaemia n = number of cells investigated

Chemography or background due to mechanical stress was never observed with this procedure

The slides were developed for 15 min in a simple developer, metol 15 g and anhydrous sodium sulphite 200 g in 2 l of distilled water. The slides were immersed for 2 min in a stop bath containing 2% acetic acid and fixed for 4 min in 8% sodium thiosulphate. They were not agitated throughout the processing as diffusion suffices for these thin emulsions. Finally the slides were rinsed twice for 10 sec in distilled water.

The cells were stained through the emulsion with Leishmann's stain. Those previously analyzed were easily found and the overlying grains counted. The background was always less than one grain per average cell area.

Cytological Classification

Myeloblasts Cells with fine leptochromatic chromatin, prominent nucleoli and a light sky blue, agranular cytoplasm.

Promyelocytes Usually larger than the blasts with a shallow depression in the nucleus, distinct nucleoli and only azurophilic granules in the basophilic cytoplasm.

Myelocytes The chromatin is slightly more condensed. Eosinophilic areas occur in the cytoplasm, which contains one or more specific granules.

Metamyelocytes The nuclear chromatin is condensed, nucleoli are absent and the cytoplasm eosinophilic with predominantly specific granules.

Mature forms If the nucleus was more than twice longer than wide it was classified as a stabform. The segmented neutrophils showed distinctly lobulated nuclei.

Results

It appears from table I that the neutrophilic myeloid cells from normal individuals and from patients with CML showed essentially the same cytological distribution of DFP ^3H uptake.

The highest mean grain count was found in promyelocytes and a somewhat lower count in myelocytes. The DFP uptake in metamyelocytes and mature forms was nearly identical and on a level about half that of the promyelocytes. Myeloblasts had very low grain counts.

The incorporation in monocytes and eosinophils was not investigated systematically, but we have found the activity of these cells to be very low, as was the uptake by lymphocytes and erythropoietic cells, barely exceeding the background values.

The standard deviation of the grain counts was considerable, particularly in promyelocytes and myelocytes. In one haematologically normal individual the mean grain count was significantly higher in segmented cells than in stab- and metamyelocytes.

Discussion

The present observations differ from those of KURTZ *et al* [8] in that promyelocytes showed heavier labelling than myelocytes in normal individuals as well as in CML patients. This discrepancy may be apparent rather than real. First, in the study by KURTZ *et al* [8] the difference between promyelocyte and myelocyte grain counts was only 6% of the peak value. Second in view of the fact that these authors found it difficult to observe the cytoplasmic granules through the photographic emulsion, some promyelocytes may have been interpreted as myelocytes. Finally, the criteria for cytological classification were not defined and may have differed from those employed in the present study.

The wide scatter of the individual grain counts within the various cytological compartments cannot entirely be due to methodological factors as cells belonging to the same cytological compartment and lying close together frequently presented widely differing grain counts.

The position of a given cell in the mitotic cycle is likely to be important in this respect as the volume of cytoplasm and the enzyme content in a premitotic G2 cell is larger than in a G1 cell, *ceteris paribus* it probably takes up more DTP-³H.

In one of the controls rising counts were observed in metamyelocytes, stab and segmented neutrophils. No clinical or technical factor has been found to explain this finding.

The DTP-enzyme complex is chemically very stable [2, 3]. It was consequently conceivable that the DTP-³H uptake could be applied for cytological classification of metaphases prepared for karyotype analysis. However, so much of the label was lost from the cells during chromosome preparation that we were not able to carry out cytological classification of the metaphases with sufficient certainty.

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Serum Haemopexin Concentration in Patients with Various Haemoglobinopathies

Effect of Splenectomy

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Abstract Serum haemopexin levels were determined by the radial immunodiffusion method in 96 patients with β thalassaemia major (27 after splenectomy) in 36 with sickle cell disease and in 19 with sickle cell thalassaemia disease (4 after splenectomy). Lowering of haemopexin concentration was extremely marked in thalassaemia major and - to a lesser extent - in sickle cell and sickle cell thalassaemia disease. Comparatively higher values were noted after splenectomy.

Key Words
Haemopexin
Sickle cell disease
Splenectomy
Thalassaemia

A number of human serum proteins have the property to bind with haemoglobin or haem. Haptoglobin, albumin and haemopexin. Haptoglobin binds with haemoglobin while albumin and haemopexin bind with haem to form methaemalbumin and haemopexin haem complexes, respectively. Haemopexin has been isolated and purified by SCHULTZE *et al* [9] in 1961. The human haemopexin contains approximately 20% carbohydrates and the molecular weight is $57\,000 \pm 3\,000$ [11]. Haemopexin binds haem in a equimolar ratio [3] and the haem haemopexin complex is subsequently eliminated from the circulation and degraded mainly in the liver [8]. In healthy individuals of both sexes serum haemopexin levels range between 500 and 1 000 $\mu\text{g/ml}$ [8]. Deficiency of haemopexin was noted in haemolytic anaemias including different haemoglobinopathies in a relatively limited number of patients [2, 6-8, 10].

The purpose of the present study was to extend these investigations in a larger number of patients and to compare the values between the non-splenectomized and splenectomized patients.

Material and Methods

Patients studied included 96 cases of β thalassaemia major (27 after splenectomy) 36 cases of sickle cell disease and 111 with sickle cell thalassaemia disease (4 after splenectomy). The age of our patients varied between 2 and 27 years. A control group of 20 sera from healthy individuals aged 5-30 years was similarly investigated.

Blood was taken by ven puncture. After separation of the cells the sera were sealed and frozen at -20°C until used. For the determination of serum haemopexin concentration the single radial immunodiffusion method was performed using commercially available immunoplates (Partigen Haemopexin Behringwerke Marburg FRG). The standard curve for the determination of the haemopexin was calculated with the aid of a standardized human serum (Behringwerke). Serum immunoelectrophoresis of GRABAR and WILLIAMS [1] was carried out on microscopical slides as described by SCHIRDIGER [12] by using specific anti human haemopexin immuniserum (Behringwerke).

Results

The mean serum haemopexin level in the 20 normal individuals was 79.20 mg/100 ml the values ranging between 69 and 88 mg/100 ml. In the 69 cases of thalassaemia without splenectomy the mean serum haemopexin level was 13.3 mg/100 ml (range 0-28.5 mg/100 ml) while in the group after splenectomy (27 cases) it was 25.2 mg/100 ml (range 15-49 mg/100 ml). The difference between the mean haemopexin levels in normal individuals and the thalassaemia group as a whole was highly significant ($p < 0.001$) as was the difference between the groups without splenectomy and after splenectomy ($p < 0.001$). In the 36 cases of sickle cell disease the mean serum haemopexin level was 28.1 mg/100 ml (range 9.5-47 mg/100 ml) while in sickle cell thalassaemia the values were 13.90 mg/100 ml (range 0-27 mg/100 ml) in non splenectomized cases and 42.5 mg/100 ml (range 40-45 mg/100 ml) in the group after splenectomy. The difference between the mean haemopexin level in normal individuals and in patients with sickle cell or sickle cell thalassaemia disease was highly significant ($p < 0.001$) as was the difference between the groups without splenectomy and after splenectomy of the latter disease ($p < 0.001$). No sex differences was found in any group. No attempt was made to correlate the severity of the clinical or haematological condition (especially the severity of the haemolytic process) to the values obtained.

Immunoelectrophoretic studies of all the sera investigated did not disclose any difference in the electrophoretic mobility of this protein in the

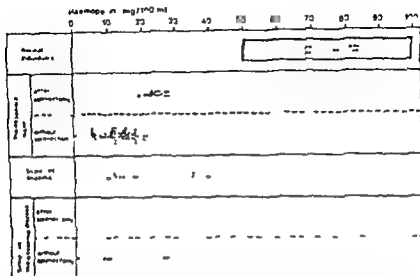


Fig. 1 Haemopexin levels in \square normal individuals, 69 non-splenectomized and 27 splenectomized patients with thalassaemia major, 36 with sickle cell anaemia and 14 non-splenectomized and 4 splenectomized patients with sickle cell thalassaemia disease.

normal individuals and at the different haemoglobinopathies. The precipitation line of the haemopexin was evident only when its concentration in the serum was greater than 20 mg/100 ml. Figure 1 illustrates the results of the individual cases.

Discussion

It has long been known that haptoglobin is depleted from the plasma of patients with haemolytic diseases because of the rapid removal of the haptoglobin-haemoglobin complexes. The serum of these patients may also have low haemopexin levels and haem-albumin complexes usually referred to as methaemalbumin. Earlier reports demonstrated the deficiency of haemopexin in hereditary haemolytic disorders by the immunoelectrophoretic technique [6]. The advent of radial immunodiffusion technique by Mancini *et al.* [5] incited a new interest on the behaviour of this protein in various haematologic disorders.

Material and Methods

Patients studied included 96 cases of β thalassaemia major (27 after splenectomy) 36 cases of sickle cell disease and 18 with sickle cell thalassaemia disease (4 after splenectomy). The age of our patients varied between 2 and 27 years. A control group of 20 sera from healthy individuals aged ≤ 30 years was similarly investigated.

Blood was taken by venipuncture. After separation of the cells the sera were sealed and frozen at -20°C until used. For the determination of serum haemopexin concentration the single radial immunodiffusion method was performed using commercially available immunoplates (Partigen Haemopexin Behringwerke Marburg FRG). The standard curve for the determination of the haemopexin was calculated with the aid of a standardized human serum (Behringwerke). Serum immunoelectrophoresis of GRAHAM and WILLIAMS [1] was carried out on microscopic slides as described by SCHIFFINOGER [12] by using specific anti human haemopexin immuniserum (Behringwerke).

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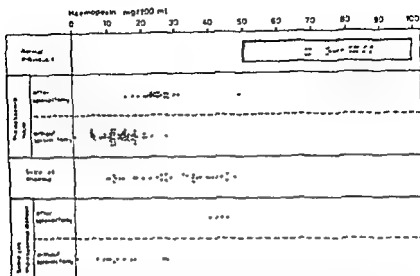


Fig 1 Haemopexin levels in 20 normal individuals, 69 non-splenectomized and 27 splenectomized patients with thalassaemia major, 36 with sickle cell anaemia and 14 non-splenectomized and 4 splenectomized patients with sickle cell thalassaemia disease

normal individuals and/or the different haemoglobinopathies. The precipitation line of the haemopexin was evident only when its concentration in the serum was greater than 20 mg/100 ml. Figure 1 illustrates the results of the individual cases.

Discussion

It has long been known that haptoglobin is depleted from the plasma of patients with haemolytic diseases because of the rapid removal of the haptoglobin-haemoglobin complexes. The serum of these patients may also have low haemopexin levels and haem albumin complexes usually referred to as methaemalbumin. Earlier reports demonstrated the deficiency of haemopexin in hereditary haemolytic disorders by the immunoelectrophoretic technique [6]. The advent of radial immunodiffusion technique by MASURET *et al* [5] incited a new interest on the behaviour of this protein in various haematologic disorders.

The mean values of serum haemopexin in the present investigation are consistent with the findings of other investigators [2, 8]. It is also known that infants approach adult values by the end of the first year [2].

In thalassaemia major, despite the limited number of patients studied, low haemopexin levels were consistently found [2, 7, 8, 10]. Low values of this protein were associated with lowering of haptoglobin concentration [10] (although not always to the same extent) and invariably with high concentration of haem [7, 10]. The latter seems to be the most decisive factor for haemopexin depletion. In the present study the mean value for serum haemopexin in non splenectomized patients with thalassaemia major was found to be 13.3 mg/100 ml. It is probable that the higher values obtained in this study are due to the different sensitivity of the immunodiffusion method used and the greater number of patients. In splenectomized patients higher values were found and the difference was statistically significant. Sera were obtained at various times after the operation. Comparable observations of early reports are rather scanty. MÜLLER-EBERHARD and CLEVÉ [6], by using immunoelectrophoresis, noted that deficiency of haemopexin persisted after splenectomy in 4 out of 5 patients and the same deficiency was observed in 9 additional cases studied at various time intervals after splenectomy.

In sickle cell disease low concentrations of serum haemopexin were also noted, comparable to those of thalassaemia major after splenectomy. Lower values (5–12.5 mg/100 ml) are reported by HANSTEIN and MÜLLER-EBERHARD in 5 patients [2], while in a more recent paper of MÜLLER-EBERHARD [8] no distinction is made between the values observed in patients with sickle cell anemia and sickle cell haemoglobin C disease.

It was found that lowering of haemopexin level below 20 mg/100 ml was associated with complete lack of its precipitation line in the immunoelectrophoresis and it can be concluded that this level represents the limit of sensitivity of this method.

From the data of the present study and from previous reports no conclusions can be drawn as to whether the reduction of haemopexin reflects exactly the degree of haemolysis. However, it can be assumed that since lowering of haemopexin concentration is associated with high concentration of haem measurement of the former may become a useful index of severity of the haemolytic process. On the contrary serum haptoglobin concentration is not considered as reliable index of the degree of haemolysis since this protein is easily depleted from the circulation even after minor haemolytic episodes [8]. Furthermore, from this study as well as

similar observations on the serum haemopexin level after the removal of the spleen in different haemolytic disorders [2, 6] one can assume that the increase of haemopexin level after splenectomy reflects a beneficial effect of this operation on the haemolytic process

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Splenic Function and Infection in Sickle Cell Anemia¹

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Abstract Splenic functions have been studied in children with sickle cell anemia by determination of ⁵¹Cr uptake and release of factor VIII after adrenaline infusion. A significant correlation between early cessation of splenic function and susceptibility to severe infections has been found.

Key Words
Infection
Sickle cell anemia
Splenic function

Children with sickle cell anemia are very prone to infection especially with pneumococcus [5-10]. In the child of pre-school age, overwhelming septicemia or meningitis is a frequent cause of death. When exposed to massive bacterial invasion these children are unable to cope with it and react like individuals who have had their spleens removed early in life. The fulminant course of these infections suggests a defect in the immune mechanism in patients with sickle cell anemia and has been related to various abnormal splenic functions [1, 8, 11, 15].

By using 2 simple, readily performed tests, we studied the phagocytic function of the spleen and measured the release of factor VIII after stress. We then attempted to determine whether a relationship existed between the susceptibility to severe bacterial infections and measurable splenic functions in patients homozygous for hemoglobin S. Our results indicate that patients at high risk for severe infections can be detected by splenic function tests.

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Materials and Methods

We studied a total of 35 children divided into 3 groups. Group 1 consisted of 21 children homozygous for hemoglobin S as determined by hemoglobin electrophoresis. Their ages ranged from 9 months to 15 years. Group 2 was formed by 10 healthy children with a hemoglobin pattern of AA whose ages ranged between 3 and 13½ years. They served as normal controls for splenic functions. Group 3 consisted of 7 patients between 3½ and 13½ years of age who had had splenectomies in the past for various reasons. They served as controls for absent splenic functions.

All children were free of infection, and the patients with sickle cell anemia were not in crisis at the time the tests were performed. The latter were selected at random from our sickle cell clinic population. All the splenectomized patients had been followed in our hematology clinic from 1 to 5 years. After completion of the tests on all children, the hospital records of the test subjects with sickle cell anemia were reviewed to determine the number of hospitalizations for severe bacterial infections (pneumonia, meningitis). Febrile illnesses without positive X ray or bacteriological findings were not counted.

The phagocytic function of the spleen was studied by the 99m technetium (^{99m}Tc) radioisotope uptake method. A freshly prepared solution of ^{99m}Tc sulfur colloid was injected intravenously at a dose of 0.006 mCi/lb of body weight. After 30 min, the scintillation image (Scintiphoto) was recorded on polaroid film with a Nuclear Chicago pho gamma III scintillation camera with a thin septum multichannel low energy collimator (Model 821742) in anterior and posterior projections. The scans were evaluated subjectively by one of us (O.S.H.) and placed in one of 3 categories: normal, decreased, or absent splenic uptake. The posterior view was found to be the most satisfactory for evaluation of the spleen. Examples of normal, decreased, and absent splenic uptake of ^{99m}Tc are given in figure 1.

The second test measured the ability of the spleen to release factor VIII after stress. Lurie *et al.* [7] have demonstrated that the spleen, serving as a storage organ for factor VIII, releases it following adrenaline infusion in normal individuals. In contrast, splenectomized persons did not achieve significant rises of factor VIII activity in their plasma. We infused adrenaline hydrochloride in a dose of 0.0042 mg/kg of body weight at a concentration of 6.6 µg/ml in physiologic saline. The calculated dose was infused by a Harvard pump in exactly 30 min. Blood samples were collected in 38% sodium citrate in a relationship of 9:1 before and immediately after the infusion. The plasma was assayed for factor VIII activity by a one stage method using partial thromboplastin time [12]. The changes in factor VIII activity after the infusion were expressed as percent rise from the pre-infusion value or if the factor VIII activity was lower following the infusion, as percent fall from the base line.

The statistical analysis of the results on the splenic release of factor VIII was carried out by the Student *t* test. The results are reported as the mean \pm one standard deviation. For comparison of the relationship between infection and splenic function, the Fisher exact test was chosen.

predisposition was attributed to the absence of the spleen, which not only acts as a filter for bacteria from the blood stream but also plays an important role in the formation of antibodies against invading organisms. Although fatalities had occurred in older children, evaluation of 467 children after splenectomy by ERAKLIS *et al* [4] showed clearly that the over all mortality from infection was about 2.5 times greater in children with splenectomy before the age of 4 years than if the operation was performed after that age. The report also demonstrated that mortality was much higher in children with basic underlying chronic disorders affecting the reticuloendothelial system, e.g., thalassemia, histiocytosis X and conditions associated with congestive splenomegaly, than in children who had been splenectomized for trauma to the spleen or congenital spherocytosis.

Although it was common knowledge that the spleens of patients with sickle cell anemia undergo progressive shrinkage and fibrosis, only recently has attention been focused upon the spleen in relationship to the increased susceptibility to infection in these patients. The studies of FRANKSON *et al* [8] of splenic uptake of ^{99m}Tc showed that a large spleen in patients with sickle cell anemia was not necessarily a functioning spleen. He also demonstrated that the function could be restored temporarily in some young children with splenomegaly following transfusion of rather large amounts of normal red cells [9]. He attributed this phenomenon of functional asplenia to sluggish blood flow in the splenic cords with the biochemical environment in the spleen facilitating sickling of the red cells. Sickled cells and increased blood viscosity inherent in homozygous hemoglobin S disease led to a relative obstruction of the blood flow through the sinusoid system, thus bypassing the phagocytic cells in the spleen. Transfusion of normal cells restored the splenic circulation and the phagocytic activity temporarily. However, in children with non palpable spleens no splenic uptake of ^{99m}Tc could be achieved after transfusions. Whether the inability to restore the phagocytic function should be attributed to anatomic asplenia resulting from splenic fibrosis or to a total functional asplenia is difficult to evaluate.

It seems that cessation of splenic function is a gradual process, based upon the occurrence of vascular occlusion and infarction and that the time within which complete functional asplenia or splenic fibrosis develops is variable. If the process progresses slowly over a number of years, exposure to and challenge with a variety of bacterial organisms might still be met by a spleen capable of functioning at least partially, thus al-

lowing the child to build up his body defenses in an approximately normal fashion. Should, however, splenic function cease very early in life, a child with sickle cell anemia and complete functional or anatomic asplenia would face the same risks as a patient splenectomized before the age of 4 years as far as morbidity and mortality from infection are concerned. The clinical importance of detecting these endangered children is obvious. By applying both splenic function tests concurrently, we feel that we have found a valid method for detection of patients at high risk for severe infections. Although release of factor VIII by the spleen is not directly involved in primary defenses against infections, the absence of a response under stress does reveal the general impairment of the functional potential of the spleen. Both tests are not only readily available and easy to perform, but can be repeated at intervals without danger to the patient. The dose of adrenaline infused does not produce undesirable side effects, such as significant rise in blood pressure and heart rate. The exposure to radiation is minimal, the amount of radiation delivered is comparable to that of an abdominal flat plate [14].

We are fully aware of the limitations of a retrospective study, but we feel that our results indicate a need for further investigation of the value of the 2 tests in detecting the cessation of splenic function in patients homozygous for hemoglobin S. Patients developing functional asplenia or splenic fibrosis at an early age might benefit from monthly injections of long-acting penicillin to prevent overwhelming pneumococcal infections.

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Ribonucleic Acid and Phytohemagglutinin on Rat Leukocyte Cultures within Diffusion Chambers

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Abstract RNA extracted from normal liver of inbred rats was added to *in vivo* syngeneic peripheral leukocyte cultures enclosed within diffusion chambers either 10 min after or 95 min before PHA. An increase in blastogenesis and DNA synthesis was observed the highest values being obtained in 72 hour cultures when RNA was added after PHA. Similar results were seen with RNA pretreated with RNase, that is, with 2S fragments. In the absence of PHA, RNA had no blastogenic effect but caused a marked increase in mononuclear phagocytic cells which could be inhibited by RNase pretreatment.

Key Words
labeled cells
PHA
Phagocytic cells
Rat leukocyte cultures
RNA

It is well known that the addition of phytohemagglutinin (PHA) to *in vitro* or *in vivo* leukocyte cultures causes morphological [3, 6, 19, 20] and biochemical lymphocyte transformation [9, 10, 14, 15]. Many substances are capable of modifying this lymphocyte transformation and the experimental evaluation of the effect of biological agents on lymphocyte-PHA responsiveness may help to elucidate the mechanism of action. The suppression of human lymphocyte transformation by adding RNA has been reported [1, 2, 17, 18]. We have also been able to observe an inhibitory effect of high doses of RNA, although low doses of RNA proved to have a stimulating effect [7].

The object of the present paper is to investigate the effect of normal syngeneic RNA on rat leukocyte cultures, both before and after PHA stimulation. The cultures were carried out *in vivo* within diffusion chambers implanted intraperitoneally.

Methods

Animals Male rats of the inbred G strain, weighing 200-300 g were used

Diffusion chambers were prepared as described by CAPALBO *et al* [4] using Lucite rings and Millipore filters of 0.22 μ m

Heparin 100 IU of heparin/ml of blood was added

Culture medium TC 199, Gibco

Phytohemagglutinin (PHA) 0.001 ml of PHA P Difco 0.1 ml of culture medium was used

RNA was extracted at 25°C by the method of TRAVNICEK *et al* [21] 50 μ g RNA 0.1 ml of culture medium was used

Labeled DNA precursors methyl thymidine 3 H (3 H TdR, New England Nuclear Corp., Boston) Specific activity 67 Ci/mM

Radioautographs as described by KOPRIVA and LEBRON [11] using Kodak NTB2 emulsion.

Leukocyte cultures For each of 3 experiments 50 rats under ether anesthesia were bled by cardiac puncture. After sedmentation, the supernatant plasma was removed and centrifuged at 1000 rpm for 15 min. The cell bottom was resuspended with TC 199 to a final concentration of 10^6 leukocytes/0.1 ml. The cultures were distributed into 42 Millipore diffusion chambers. No antibiotics were added. Six diffusion chambers corresponding to each group were introduced into the peritoneal cavity of 2 syngeneic male rats. Three chambers from each group (within 1 rat) were removed after 72 h and 3 after 96 h. They were treated with pronase - in order to dissolve the clot -, opened up, their content pooled (for each group) centrifuged and washed once with saline solution. The cell bottom was resuspended in 1 ml TC 199 and pulsed with 1 μ Ci/ml 3 H TdR, for 1 h.

One thousand cells were counted in the radioautographies of each sample. Whenever mononuclear phagocytic cells (MPC) were observed, they were counted separately from the lymphoid cells. The percentage of blast transformation, MPC with and without phagocytosis, labeled cells and mitotic index were determined.

The whole experiment was repeated 3 times.

The χ^2 test was used for statistical analysis.

Results

The 3 different experiments gave very similar results. These have been averaged and are summarized in table I, being subdivided into 72- and 96-hour cultures.

Discussion

Attempts to culture rat peripheral leukocytes have not always led to success [6-10]. However, these cells have been reported to grow once

Table 1 The addition of RNA 10 min after or 95 min before rat leukocyte cultures were stimulated with PHA. Results of 72- and 96-hour cultures *in vitro* within diffusion chambers (in %)

Leukocyte cultures with	72 hour cultures						96-hour cultures					
	B	LC	Ly	MPC	LC	MI	B	LC	Ly	MPC	LC	MI
1 PHA	55	21	43	2	0	2	59	15	39	2	0	1.5
2 PHA+RNA	86	43	14	0	0	1.9	58	5	42	0	0	1
3 PHA+RNA +RNAse	82	44	18	0	0	2.5	54	0.7	46	0	0	0.9
4 RNA	0	0	72	28	0	0	0	0	78	22	14	0
5 RNA+RNAse	0	0	98	2	0.3	0	0	0	98	2	0.2	0
6 RNA+PHA	66	24	34	0	0	2.6	69	21	30	1	0.6	0.9
7 RNA+RNAse +PHA	66	30	33	1	0	1	63	23	36	0.6	0	0.7

B = blast cells, LC = labeled cells, Ly = lymphocytes, MPC = mononuclear phagocytic cells, MI = mitotic index

stimulated with PHA, both *in vitro* [16], or *in vivo* [8]. In the present experiments, it has been possible to obtain high values of blast transformation, 55 and 59%, and mitotic index, 1.5 and 2, after 72 and 96 h of PHA stimulation, respectively. These results have not been reached easily and are the culmination of a long series of experiments which led to the development of this simple, reproducible, *in vivo* technique for culturing leukocytes, without the use of antibiotics.

When syngeneic liver RNA was added to rat leukocyte cultures 10 min *after* PHA, a marked increase in blastogenesis, up to 86%, was observed in 72 hour cultures, as well as an increase in DNA synthesis, evidenced by 43% of labeled cells. Previous incubation of RNA with RNAse did not modify these results. There is no doubt that RNAse pretreatment was effective since the sucrose gradient analysis revealed that the total RNA content had been degraded in 2S fragments of polynucleotides. Apparently, 2S fragments would be as active as polymerized RNA in increasing PHA lymphocyte stimulation. The explanation of these results is not easy. The site of RNA action is unknown, it has been reported to be present on the cell surface as well as in the cytoplasm and nucleus of these cells [12, 13]. According to KAY [9], during PHA stimulation, there

■ an alteration in the proportion of the different types of RNA synthesized and an increased rate of ribosomal and possibly of transfer RNA. It could be that RNA and/or its degradation products potentiate the action of PHA on the cell surface, or even that they act as raw material, facilitating an increase in RNA synthesis within the cell. This would be accompanied by an increased DNA synthesis which would explain the higher number of ^3H TdR labeled cells in the corresponding groups.

In 96-hour cultures, both RNA and RNA pretreated with RNase groups had returned to values similar to that of PHA-stimulated controls, but DNA synthesis was much lower. It would seem as if the optimal RNA stimulation had occurred around 72 h after the cells become exhausted with the consequent drop in mitogenesis.

When RNA was added 95 min before PHA, again both with and without RNase pretreatment an increase in blastogenesis was observed, as compared to PHA controls in 72- as well as in 96-hour cultures. In this case, the peak values of 86% observed in 72 hour cultures when RNA was added after PHA were not reached. On the other hand in 96 hour cultures, DNA synthesis remained high, as if the cells had not become exhausted. These results indicate that the addition of RNA before PHA stimulation is quantitatively less effective but more lasting than RNA added after PHA stimulation.

In the absence of PHA, RNA had no stimulating effect, no blast transformation or mitosis being observed. It is interesting to note, however, that mononuclear phagocytic cells (MPC) reached 28% of the cell count in 72 hour cultures and remained high at 96 h. Some of these cells had phagocytized autologous red blood cells. Almost no labeling or mitotic figures were seen. Cytochemically MPC were peroxidase negative which helped to discard any morphologically doubtful cell especially from those of granulocytic origin. The number of MPC decreased to normal values, down to 2%, when RNA was previously incubated with RNase, thus indicating that only polymerized RNA is active in this case and confirming at the same time the destructive effect of the enzyme.

It can be concluded that (1) the addition of syngeneic normal RNA to *in vitro* rat peripheral leukocyte cultures potentiates the stimulating effect of PHA; this effect is quantitatively greater although short lived when RNA is added 10 min after PHA than when it is incorporated 95 min before PHA; (2) The same results are obtained with RNA pretreated with RNase indicating that 2S fragments would be responsible for this effect; (3) In the absence of PHA RNA has no blastogenic effect but causes an

increase in MPC, which disappear upon RNase pretreatment. In this case, polymerized RNA would be required and the mechanism of action can be said to be different in the presence or absence of PHA.

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Study of the Effect of Actinomycin D on the Thrombocytopoiesis of Mice, Using ^{75}Se -Labelled Methionine

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Abstract After a single administration of 15 μg actinomycin D subcutaneously, the platelet count of mice fell markedly on the 5th day. At this time thrombopoietic activity can be demonstrated in their serum. This is manifested in the increase of the platelet count of recipient animals and in the enhancement of the incorporation of ^{75}Se methionine.

Key Words
Actinomycin D
Protein synthesis
Selenomethionine
Thrombocytopoiesis

On the basis of previous investigations it could be presumed that in the regulation of the platelet count an important role is played by a factor or factors either of a protein nature or bound to protein [3, 6, 9, 14, 15].

In recent years inhibition of protein formation by actinomycin D (Act D) has been widely recognized. This effect is exerted via the inhibition of RNA synthesis. The mechanism and specificity of the effect are discussed by GELIERT *et al* [7].

It appeared reasonable to examine how the platelet count and thrombocytopoiesis of experimental animals change on the administration of Act D.

Methods

The experiments were carried out on mice of the C57BL strain of both sexes with an average weight of 20 g, maintained on a standard diet. The platelet count was obtained by a direct phase-contrast microscopic method [5]. The method of PRINGTON [11] was used to study the incorporation of methionine labelled with the radioactive ^{75}Se (^{75}SeM). Measurement was performed with an automatic sample changer connected to a Packard Tri-carb spectrometer. The only differences from the

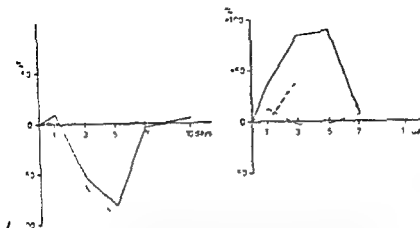


Fig. 1 Effects of various doses of Act D on the platelet count of mice. — = 10 μ g Act D (5 mice), — — = 15 μ g Act D (15 mice), - - - = 25 μ g Act D (5 mice).

Fig. 2 Effects of serum of mice treated with 15 μ g Act D on the platelet counts of normal recipients. — = 48 h (5 mice), - - = 72 h (15 mice), — — — = 5 days (15 mice) and — — — — = 7 days (5 mice) after Act D injection.

The usual method of PRAXIDON was that the material was administered intraperitoneally (the results of parallel ip and iv studies were the same) while blood was obtained from the mice by heart puncture (but from the peritoneal cavity) transverse of the abdominal aorta.

Results

In one case different doses of Act D (10, 15 and 25 μ g s.c.) were administered to three groups. The platelet counts were determined at 4 times given in figure 1. All of the animals to which 25 μ g were administered died on the 5th day. The curves give the average percentage changes of the platelet counts of the mice in the individual groups. After the administration of 15 μ g Act D marked thrombopenia was observed on the 4th day and the animals remained alive. Experiments were also carried out later with similar doses. Figure 2 shows the effects of the serum of mice treated with such doses on the platelet counts of normal recipients. Here too the results are given as average percentage changes. Transfer of serum 5 days after the administration of Act D led to the most pronounced thrombopenia in the normal animals, likewise on

3

Injection schedule	³⁵ Se meth 0.2 μC p	³⁵ Se utilization, % × 10 ²			
		n	n	n	n
15 μg Act D s.c.		0019 ±0.00049	0077 ±0.01233	0117 ±0.03464	
Control		0057 ±0.00364	0062 ±0.00497	0063 ±0.00497	
Days	-1, 0	+2	+3	+5	

4

Injection schedule	³⁵ Se meth 0.2 μC p	³⁵ Se utilization, % × 10 ²			
		n	n	n	n
0.3 ml serum of Act D treated mice		0073 ±0.00878	0177 ±0.0107	0146 ±0.03014	
0.3 ml normal mouse serum		0062 ±0.00494	0076 ±0.00667	0165 ±0.01378	
Days	-1, 0	+2	+3	+5	

5

Injection schedule	³⁵ Se meth 0.2 μC p	³⁵ Se utilization, % × 10 ²			
		n	n	n	n
0.3 ml/day of Act D treated animals		0055 ±0.0110	0700 ±0.016	0173 ±0.0140	
0.3 ml/day normal sera		0078 ±0.0107	0070 ±0.0065	0073 ±0.0065	
Days	-1, 0	+1	+3	+5	

Fig. 3 Effect of Act D on the ³⁵Se-methionine utilization of blood platelets of mice

Fig. 4 Effect of serum of mice treated with Act D on the ³⁵Se utilization of the blood platelets of normal recipients

Fig. 5 ³⁵Se utilization in blood platelets of normal animals following the repeated administration of sera from Act D treated mice

5th day after the administration of the serum (0.3 ml per mouse i.p.) when the platelet count of the donor mice was the lowest. Sera obtained 48 and 72 h after the Act D administration also possessed activity, while the serum taken on the 7th day essentially did not change the platelet counts of the recipients.

In further experiments the direct effect of Act D and the effect of the serum of mice treated with it on the platelet count were investigated with the incorporation of SeM. Figure 3 shows the effect of Act D administered 48 h before the SeM. The utilization after 48 h is substantially lower, but that on the 5th day significantly higher than for the controls (which were treated only with SeM). The 5th day after the SeM corresponds to the 8th day following the administration of Act D. At this point the platelet count of the animals already exhibits a marked increase (fig. 1).

A similar effect was also detected after the administration of the serum of mice treated with Act D. The serum, which was obtained on the 5th day after administration of Act D, was given to normal mice. The serum of mice treated with Act. D 48 h prior to the ScM exhibited a significant utilization increase on the 3rd day after the labelling compared to that of mice treated with normal mouse serum (fig. 4). The difference was even more considerable if the sera of normal mice and those treated with Act. D were administered repeatedly for 3 days before the ScM (fig. 5).

Discussion

Numerous publications deal with the protein synthesis inhibiting effect of Act D. According to the investigations of KAY [8], it also inhibits the incorporation of ^{14}C labelled leucine into the lymphocytes in spite of phytohaemagglutinin. BAKKE and LAWRENCE [1] demonstrated its inhibitory effect on TSH and thyroxine formation. Similar results were reported by FLEISCHER and BATTERBEE [6] in connection with ACTH formation. With this method PENNINGTON [12] demonstrated the thrombopoietic activity of the serum of thrombocytopenic patients. His investigations indicated that the utilization was also proportional to the amount of plasma given: the utilization increased after the administration of larger and repeated doses. The method is generally accepted for the measurement of thrombopoietic activity [13]. It is very important to choose the correct time interval between the times of intervention (the administration of Act D or the transfer of the serum of the animals treated with this drug) administration of ScM and the measurement of the activity in the thrombocytes, taking into consideration the time of formation and the life time of the blood platelets. The 24th, 48th and 72nd hours after the administration of ScM seemed the most suitable, but in certain cases a significant difference can be found even on the 5th day. COOPER *et al.* [2] used ^{35}S labelled sodium sulphate with which they demonstrated a thrombopoietic effect in healthy animals with the serum of thrombopenic animals (mice and rats). Studies with ^{35}S were also carried out by LAWRENCE and HARRIS [10] on rats subjected to exchange transfusion and hypertransfusion. There was a marked activity increase in the former on the 4th day.

Our studies with Act D too seem to support the view that factors of protein nature play an important role in thrombocytopoiesis and the regulation of the platelet count. It is clear that with the cessation of the inhibi-

3

Injection schedule	⁷⁵ Se meth. 0.2 μC p.	⁷⁵ Se utilization % ± 10 ²					
15 μg Act D sc		0019 ±000	n 6	0007 ±001	n 9	0117 ±014	n 9
Control		0055 ±0003	n 6	0062 ±003	n 9	0063 ±0006	n 9
Days	-2 -1 0	+2		+3		+5	

4

Injection schedule	⁷⁵ Se meth. 0.2 μC p.	⁷⁵ Se utilization % ± 10 ²					
0.3 ml serum of Act D treated animals		0073 ±0008	n 9	0127 ±0037	n 9	0146 ±0035	n 9
0.3 ml normal mouse serum		0062 ±0004	n 3	0046 ±0014	n 3	0155 ±0036	n 4
Days	-2 -1 0	+2		+3		+5	

5

Injection schedule	⁷⁵ Se meth. 0.2 μC p.	⁷⁵ Se utilization % ± 10 ²					
0.3 ml/day of Act D treated animals		0055 ±0013	n 9	0100 ±017	n 8	0173 ±0050	n 9
0.3 ml/day normal sera		0059 ±0006	n 5	0076 ±0053	n 11	0090 ±0069	n 11
Days	-2 -1 0	+1		+3		+5	

Fig 3 Effect of Act D on the ⁷⁵Se-methionine utilization of blood platelets of mice

Fig 4 Effect of serum of mice treated with Act D on the ⁷⁵Se utilization of the blood platelets of normal recipients

Fig 5 ⁷⁵Se utilization in blood platelets of normal animals following the repeated administration of sera from Act D treated mice

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Our studies with Act. D too seem to support the view that factors of protein nature play an important role in thrombocytopoiesis and the regulation of the platelet count. It is clear that with the cessation of the inhibi-

tion of the protein synthesis the thrombopenia after the administration of the drug at the lowest point of which the serum of the animal possesses a marked thrombocythremic effect may be connected with a renewed protein formation. The increased uptake indicating enhanced formation and the change of the platelet count confirm this.

Acknowledgement The author wishes to express his thanks to Dr L. CSERNAI, Mrs I. LÁSZLÓ and ÁGNES BÖRÖCZ for their valuable technical assistance.

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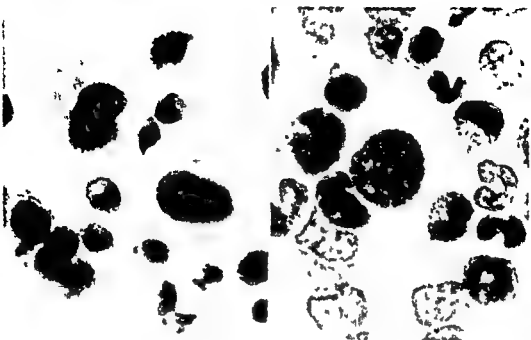


Abb 2 Unreife zellige Pannmyelose Sogenannte Mikrokaryozyten im Sternalpunktat Pappenheim

mit Maximalamplitude von 67 Prothrombin 100%, Eisenspiegel $189 \mu\text{g}^{\text{g}}$. Im Sternalpunktat vom 25. Juli 1972 (Abb 2) ergab sich im wesentlichen keine Befundänderung. Im Vordergrund standen nach wie vor mengenmässige Zunahme und Atypien der Megakaryozyten.

Bei weiterhin hochgradiger und durch Prednisolon unbeeinflussbarer Thrombozytopenie wurden am 7. August 1972 erstmals Paraleukoblasten im peripheren Blutaussstrich nachgewiesen (Gesamtleukozytenzahl 2400 mm^3 ; davon 27% Paraleukoblasten). Das Sternalpunktat vom 8. August 1972 (Abb 3-4) zeigte ein sehr polymorphes Markbild. In grosser Ausdehnung sah man Verbände unreifer atypischer blastenartiger Zellen mit basophilem Plasma und lockerer Kernstruktur. Normale granulopoetische Zellen waren stark vermindert. Die Zellen der roten Reihe zeigten zum grossen Teil eine megaloblastäre Reifungshemmung mit aufgelockertem Kernchromatin und weitem Plasmasaum. Megakaryozyten waren wie bei allen bisherigen Untersuchungen stark vermehrt und wiesen Zeichen der Reifungsstörung sowie Zellatypien auf.

Zytochemische Untersuchungen am Sternalpunktat PAS-Färbung (Abb 5) in einem grossen Teil der Blasten deutlich granular positive Reaktion, zum Teil mit mehrreihiger Anordnung der Granula. In etwa 10% der Erythroblasten und megaloblastoiden Zellen stark diffus positive Plasmareaktion. Gehalt der Megakaryozyten an PAS-positiver Substanz wechselhaft, im allgemeinen deutlich vermindert.

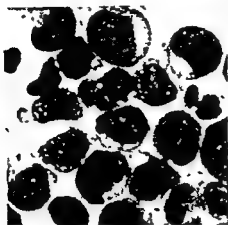


Abb 3 Untereizellige Panmyelose Paraneoplastische Mastzelleninfiltrate im Sternalpunktat Pappenheim

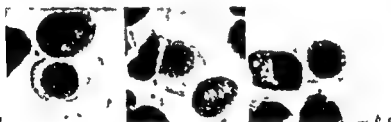


Abb 4 Untereizellige Panmyelose Megaklasten im Sternalpunktat Pappenheim

Leucina blues im Markretikulum in mäßiger Menge Eisenpigment nachweisbar kaum Sichelzellen

Saure Phosphatase (Abb 6) normale Aktivität in den Zellen der Restgranulopoese Intensive diffuse Plasmareaktion in den Blasen Entschriebene paranukleäre Fermentaktivität in einem Teil der Megaklasten Hohe Aktivität in den meisten Megakaryozyten

α-Naphthylazetatsäure (Abb 7) In einem Teil der Blasen mittelstarke bis starke Enzymaktivität mit diffuser Verteilung im Zytoplasma Nur in ganz vereinzelten Erythroblasten Enzymaktivität nachweisbar Megakaryozyten wechselhaft positiv

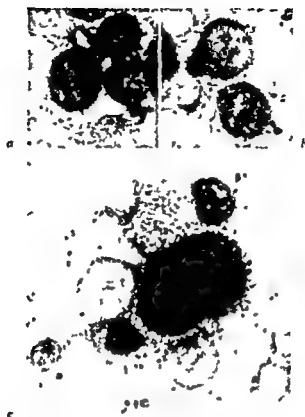


Abb 5 Unreife zellige Panmyelose Paraleukoblasten mit granular positiver Reaktion (a b) und atypische Megakaryozyten mit vermindertem Glykogengehalt Sternalpunktat PAS

Naphthol AS Azetat Esterase in einem Teil der Blasten granular positive Reaktion durch Natriumfluorid völlig hemmbar

Naphthol AS D Chlorazetat Esterase (Abb 8) neben den Zellen der Restgranulopoese lediglich Darstellung eines kleinen offenbar promyelocytar differenzierten Anteils der Blasten

Peroxydase nur in wenigen Blasten fermentaktivität nachweisbar

β -Glukuronidase alle Blasten negativ

Therapie und Verlauf Die Behandlung erfolgte mit verschiedenen Zytostatika Kombinationen (L-Asparaginase Vincristin Daunoblastin Amethopterin) in Verbindung mit Prednisolon Antibiotika und Frischbluttransfusionen Eine Remission war jedoch nicht zu erzielen Der Patient verstarb bei unbeeinflussbarer Granulozytopenie und Thrombozytopenie in der 20. Behandlungswoche Im postmortal gewonnenen Milz-

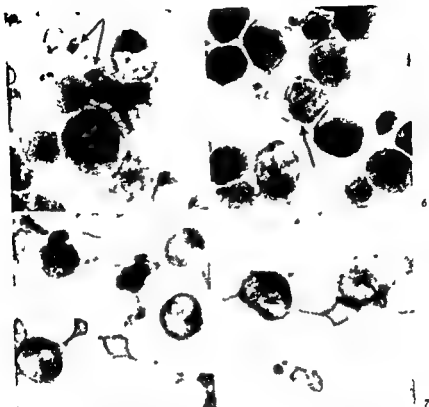


Abb 6 Unreife Panmyelose Intensive diffuse Mastreaktion in den Paraleukblasten und umförmige paranukleäre Reaktion in den Megaloblasten (→) Sternalpunktat Saure Phosphatase

Abb 7 Unreife Panmyelose Blastenpopulation mit bisher teils fehlen der Fermentaktivität Sternalpunktat α -Naphthylazetat Esterase

punktat (Abb 9) beeindruckte ein buntes Gewebsbild, das die Proliferation verschiedener Zellformen erkennen lieSS

II. Diskussion

Auffalligstes zytologisches Merkmal war bereits im ersten Sternalpunktat zum Zeitpunkt der klinischen Manifestation der Erkrankung

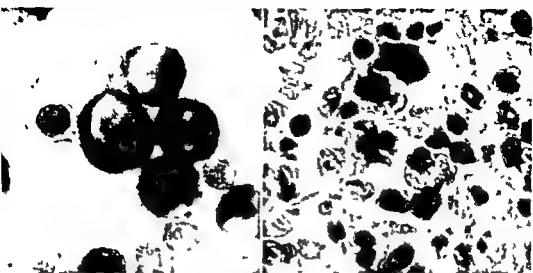


Abb 8 Unreife zellige Panmyelose: Blasteninfiltrat mit mehreren promyelozytar differenzierten Paraleukoblasten: Sternalpunktat: Naphthol ASD Chlorazetat Esterase

Abb 9 Unreife zellige Panmyelose: Milzpunktat (postmortell): HE.

eine ausgeprägte Megakaryozytose mit Zellpolymorphie und -atypien. Dieser Befund war während des ganzen Krankheitsverlaufs bis zum Tode ebenso konstant nachweisbar wie die deutliche Megaloblastose. Blasten traten anfangs nur vereinzelt und verstreut auf und waren daher in ihrer biologischen Wertigkeit zunächst nicht sicher deutbar. Auch als später Paraleukoblasten ausgeschwemmt wurden und somit der periphere Blutausstrich für eine unreife zellige Leukose sprach, bestand im Knochenmark nicht das uniforme Bild einer diffusen leukämischen Infiltration. Auch bei zytomorphologisch typischen unreife zelligen Leukosen ist die maligne Entartung durchaus nicht immer auf das leukopoetische System beschränkt. Proliferationskinetische Untersuchungen [4] zeigen vielmehr, dass sich auch die roten Vorstufen an der Reifestörung beteiligen und dass demnach die Anämie nicht allein mit einer Verdrängung der Erythropoese zu erklären ist. Schon die gewöhnliche lichtmikroskopische Betrachtung des Sternalmarkausstrichs lässt diese Reifungshemmung erkennen. Wenn man von therapiededingsnten Megaloblastosen absieht, wie sie beispielsweise nach Applikation des Antimetaboliten Cytosinarabinsid zu sehen sind [15], kann man recht häufig auch Erythroblasten mit spontan aufgetretener Reifungsdissoziation zwischen Kern

und Plasma [14] und mit allen Übergängen bis zu typischen Megakaryoblasten als Einzelexemplare sehen. Selten handelt es sich sogar um quantitativ erhebliche sekundäre Megakaryoblastosen mit perniziosaaähnlichem Markbild [5, 12, 13]. Der für dieses Phänomen mitunter gebrauchte Begriff der «Aufbrauchperniziosa» ist schlecht gewählt, weil es sich nicht um Folgen eines gesteigerten Vitamin B₁₂- und Folsäureverbrauchs durch die wuchernden Paraleukoblasten handelt, sondern um eine dem Leukoseprozess parallel verlaufende Ausreifungsstörung im erythropoetischen System. Das geht schon daraus hervor, dass sich die Veränderungen gegenüber einer antiperniziösen Behandlung als refraktär erweisen.

Wesentlich seltener sieht man bei unreife zelligen Leukosen auch eine Reifungshemmung der Megakaryozyten [1, 12], die ja in der Regel hochgradig vermindert sind. Diese kleinen, nur noch an der typischen Färbbarkeit und Struktur des Zytoplasma als Varianten des Megakaryozyten erkennbaren sogenannten «Mikrokaryozyten», die oft kaum größer als Monozyten sind und meist einen unsegmentierten runden oder ovalen Kern haben, sind aber ein sehr häufiger, fast regelmässiger anzutreffender Befund bei der chronischen Myelose [1].

In unserem Falle waren die pathologischen Veränderungen am erythropoetischen und megakaryozytären System qualitativ und quantitativ so ausgeprägt, dass man nicht von Begleiterscheinungen einer unreife zelligen Leukose sprechen kann, sondern eine gleichgeordnete maligne Entartung aller drei blutbildenden Zellreihen annehmen muss, zumal die Atypien der Megakaryopoese zeitlich eher fassbar waren als die der Leukopoese. Mit der von LÖFFLER und LEBER [6] publizierten Beobachtung einer Panmyelose besteht zytologisch weitgehende Übereinstimmung. Zytochemische Unterschiede betreffen vor allem die Erythroblasten. Diese zeigten bei LÖFFLER und LEBER in einem wesentlich höheren Prozentsatz Siderosomen und wiesen eine hohe Aktivität an α -Naphthylazetatsäureesterase auf. In unserem Falle waren von den Befunden, die für eine maligne Entartung der Erythropoese kennzeichnend sind [2, 6, 7, 10], nur an einem Teil der Zellen die Reaktionsausfälle der sauren Phosphatase und der PAS-Färbung typisch.

Die Paraleukoblasten liessen sich nach ihrem zytochemischen Verhalten nicht in die üblichen Klassifizierungsschemata unreife zelliger Leukosen einordnen und waren auch untereinander different. Unter Hinweis auf eine ähnliche Beobachtung zytochemisch heterogener Blasten [9] mag die Frage berechtigt sein, ob diese Zellen überhaupt aus einer einheitlichen Zellpopulation hervorgegangen sein könnten.

Zur Terminologie wäre anzumerken, dass der Begriff «Panmyelose» [8] missverständlich ist, da er auch für die Hyperplasie aller 3 hämatopoetischen Systeme im Knochenmark bei Polycythaemia vera gebraucht wurde [3]. Durch den Zusatz «unreiszellig» ist die Erkrankung zytologisch, nosologisch und zugleich prognostisch hinreichend charakterisiert.

Therapeutische Erfahrungen bestehen angesichts der extremen Seltenheit dieser Hamoblastoseform nicht. Da klinische Symptomatik, Verlauf und Prognose Unterschiede zu unreiszelligen Leukosen nicht erkennen lassen, sollten auch die dort geltenden Behandlungsgrundsätze angewandt werden. Auf zwei Gesichtspunkte, die den Therapieplan betreffen, sei abschliessend hingewiesen. Einmal sollte eine ätiologisch unklare Megakaryozytose mit Zellatypien als erstes Hinweiszeichen auf eine Hamoblastose ernst genommen und durch kurzfristig wiederholte Sternalpunktionen im Verlauf verfolgt werden, damit der Beginn der zytostatischen Therapie nicht verzögert wird. Die beschriebenen Atypien kommen nur in ganz wenigen Fällen bei nicht leukämischen Erkrankungen vor [1]. Ferner sollte eine Megakaryoblastoidie bei hämatologischen Systemerkrankungen nicht zur Vitamin-B₁₂-Therapie verleiten. Diese ist nicht nur wirkungslos, sondern kontraindiziert, da ja die DNS-Bildung gerade blockiert werden soll [11].

Zusammenfassung

Es wird über klinisches Bild, zytologische und zytochemische Befunde einer seltenen Form einer unreiszelligen Hamoblastose berichtet, bei der eine maligne Entartung der Leuko-, Erythro- und Megakaryopoese vorlag.

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Table 1 Hematologic findings (values in parentheses indicate our normals)

	Patient	Mother	Father	Brother
Hb g%.	6.12	12.21	10.96	10.16
Ht %.	19	38	35	-
Reticulocytes %.	2.6	-	-	-
Leukocytes per mm ³	30,600	-	-	-
Schilling test	neg.	neg.	neg.	neg.
Coombs test	neg.	-	-	-
Autohemolysis ¹	normal	normal	normal	normal
Erythrocyte fragility	markedly decreased	decreased	decreased	decreased
Plasma hemoglobin mg% (1-5 mg%)	12	-	-	-
Serum iron μ g%.	344	93 ²	206	-
Serum iron binding capacity μ g%.	362	346	304	-
Inclusion bodies (3) ³	+++	+	rare	rare
Hb A ₁ % (1.9-3%)	2.35	1.3 ²	4.72	5.1
Hb F % (2%)	14.5	2.3	3.4	3.6

¹ With and without glucose

² She was 7 months pregnant

³ Intraerythrocytic inclusion bodies appeared after incubation with 1% brilliant cresyl blue for 30 min at 37°C

that at 7 months of age he was found to be severely anemic in another hospital and was given several transfusions.

On examination he appeared pale, chronically ill, and underdeveloped (height 87 cm, weight 31.5 kg, both below the third percentile). The liver extended 2.5 cm and the spleen 4 cm below the costal margins. Other physical findings were unremarkable and no facial or skull deformities or jaundice were noted.

Laboratory examination revealed marked anemia and stained blood films showed hypochromic, polychromatic, polychromasia, and a few target and nucleated red blood cells (1% of 100 WBC). Additional hematologic data including that of family members, are given in Table 1. An alkali hemoglobin electrophoresis (pH 8.6) revealed a minor fast component in addition to Hb A and Hb A₁. On agar gel (pH 8.6) phosphate an elevated Hb F component was separated from the major fraction (Fig. 3). Scarb gel electrophoresis at pH 7 phosphate buffers indicated the presence of an anodal band corresponding to Hb H in position. Serum protein and repeated urine analyses were normal.

The parents were found to be heterozygous for de novo cross-reacting non- α chains and at 1½, 1½, and 2 years of age had manifestations similar to the patient and a 7-year-old brother received 2 blood transfusions because of polycythemia. The mother, father, and the brother have a normal Hb electrophoretic

Hemoglobin H- β -Thalassemia

S. ÖZSOYLU, G. HIÇSÖNMEZ and C. ALTAY

Hacettepe University School of Medicine and
Hacettepe Children's Hospital Medical Center, Ankara

Abstract A Turkish child with Hb H β thalassemia and a clinical picture of thalassemia major, is described. His father and brother have α β thalassemia with hematologic findings no more severe than would be expected if they had either trait alone, and his mother has α thalassemia alone. History revealed that 3 siblings have died from a similar illness in early infancy, perhaps indicating the severity of the combination in this family.

Key Words

Hb H β thalassemia
Hemoglobinopathies
Thalassemia

The thalassemias are characterized by a discordant rate of synthesis of structurally normal polypeptide chains, resulting in a deficiency of the hemoglobin, associated with the chain involved. So far α -, β - and δ -thalassemias, and their combinations, are well recognized, γ -thalassemia has been hypothesized [1] and a case of γ - β -thalassemia has been well documented [2].

Here, we are reporting a child with thalassemia syndrome, whose hemoglobin and family studies are most compatible with the diagnosis of Hb H- β -thalassemia, not previously reported to our knowledge.

Patient Report

A1 (HCH 261,514) a 3-year-old boy from the southern part of Turkey, was admitted to Hacettepe Children's Hospital Medical Center with complaints of pallor, lassitude and puffiness of the eyes since early infancy. Past history disclosed

pattern but Hb A₂ values of the mother was definitely decreased and the father's and brother's were elevated.

Methods

Hemograms were obtained by the standard techniques. Hemoglobin (Hb) concentrations were determined by the cyanmethemoglobin method and the microhematocrit was used for measuring packed cell volume. Intracrythrocytic inclusion stain, autohemolysis (with and without glucose) and erythrocytic osmotic fragility were done as described by DACE and LEWIS [3] and the sickling test by using 2% sodium metabisulfite. Serum iron, total iron binding capacity and plasma hemoglobin concentrations were determined according to STAMOND [4], PETERS *et al* [5] and CHIRBY and FLINN [6] respectively. Starch gel and agar gel electrophoresis were performed by the methods of SAMMAR [7] and ROBINSON *et al* [8] successively. Hb F values were measured by the alkali denaturation method of SINGER *et al* [9] and quantitative estimation of Hb A₂ was obtained by chromatography on α -ethylaminomethyl cellulose [10].

Comments

Thalassemia syndromes are not rare in Turkey. 137 electrophoretically-studied Cooley's anemia cases were collected at this institution between 1963 and 1971 [11]. Although α thalassemia is much less common, 4 families with Hb H disease [12] and 2 families with sickle-cell- α -thalassemia combination have been observed.

Although the pathogenesis of thalassemias has been discussed on the messenger RNA level [13-14] there is still no single practical test to establish and/or differentiate the specific type. The differential diagnosis of thalassemias remains largely dependent upon family studies. The most interesting aspect of this family is the observation of different combinations of α - and β thalassemias. The probandus had Hb H, which indicates his double heterozygosity for α -thalassemia (α -thal, + α -thal). He differs from other patients with Hb H disease in severity of anemia and elevation of Hb F. Hb F measured by the method of SINGER *et al* [9] may include not only fetal hemoglobin, but some proportion of Bart's hemoglobin since it is also alkali resistant [15]. But elevated Hb F was also shown by agar gel electrophoresis. Since the father carries a β thalassemia gene indicated by elevated Hb A₂, the increased Hb F level of the patient is explained by the presence of the β thalassemia gene. The father must also be carrying the α -thalassemia gene since the probandus has Hb H. Rare inclusion bodies seen in the erythrocytes are additional



Fig 1 Agar gel (pH 6.45) hemoglobin electrophoresis (from top) a Thalassaemia major hemolyzate only Hb F present b A I (the propositus) Hb A and elevated Hb F c-e Father mother and the brother's hemolyzates showing Hb A and a normal quantity of Hb F respectively

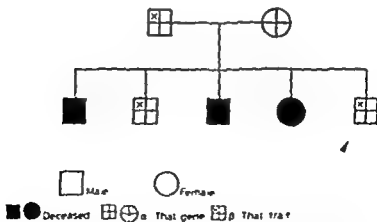


Fig 2 The family pedigree (the arrow indicates the propositus)

pattern but Hb A₂ values of the mother was definitely decreased and the father's and brother's were elevated.

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evidence of the presence of α thalassemia in the father, who has $\alpha_1\alpha_2$ -thalassemia

To produce a child with Hb H disease, the mother must have the α -thalassemia gene. This was confirmed by the inclusion bodies in her erythrocytes. Her decreased Hb A₂ level can be related to α thalassemia since iron deficiency anemia is ruled out. The elevated Hb A₂ level in the patient's brother could indicated β thalassemia trait. Because of the rare inclusion body in his smear, we believe that he, like his father has $\alpha\beta$ -thalassemia. Although Hb A₂ can be normal or decreased in patients with α -thalassemia, it is interesting that the father's and the brother's Hb A₂ level was around 5%, corresponding to reported values of Greek [16, 17], Chinese [18, 19], Thai [20], and Italian [19] double heterozygous persons. Hb A₂ elevation in Negro $\alpha\beta$ -thalassemia cases was equivocal [21]. As FESSAS [16] has pointed out, no additive effect of these 2 ($\alpha + \beta$) genes was observed in father's and brother's hematologic findings.

WASI *et al* [20] have observed patients with major hemoglobin types of Bart's and A in whom evidence suggests the presence of the β thalassemia gene in addition to double α -thalassemia with the clinical picture of thalassemia intermedia. In view of the hypothesis of multiple α -chain loci for human hemoglobins [22], we prefer the theory postulated by LEHMANN [23, 24] to explain the variety of expression of α -thalassemia in these families. Using Lehmann's theory, not only the hemoglobin picture of the propositus, but the clinical severity of the disease in this family (as indicated by the death of 3 siblings apparently from the same disorder in early infancy and the persistence of chronic severe hemolysis in the propositus) be differentiated from the cases of WASI *et al* [20]. Then, as indicated by the family pedigree (fig 2) our patient had 3 α -thalassemia genes, and the patients of WASI *et al* [20] probably had 2. If the relative rate of α - and β -chain synthesis in his and his parents' reticulocytes could be estimated by the extent of incorporation of [¹⁴C]-L-leucine studies [17, 19] the extent of $\alpha\beta$ -imbalance would be more definite.

The difference in the clinical expression of the disease in this family, may also be explained by the heterogeneity of β -thalassemia but so far no supporting evidence could be obtained.

Acknowledgments We are grateful to Dr. P. MEISER and Dr. H. WEATHERALL for their helpful discussion and Mrs. B. FROYAN for editorial help.

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Transplantation Reviews, vol II and 12 Munksgaard Copenhagen 1972 173 and 132 pp., dkkr 136- and 110-

In vitro lymphocyte stimulation is a widely used tool for genetic studies, basic investigations concerning lymphocyte physiology and for appreciation of immune reactivity in man. Many reviews have been written already on these topics in the past but progress is fast and far reaching. Therefore, the two new volumes of *Transplantation Reviews* (11 and 12) dealing with lymphocyte stimulation are very welcome. Both volumes contain reviews and progress reports by leading investigators in the field with the main emphasis on their own work.

Volume 11 (11) has seven papers on lymphocyte activation by unspecific mitogens. The first paper covers RNA metabolism following activation (H. L. CHAPLIN) followed by a report on certain morphological and functional aspects of activation in man (J. D. DOWRIE). The remaining five papers treat the large field of differential stimulation and cooperation of T and B cells in relation to membrane receptors for mitogens (J. D. STONE, ANDERSON *et al.*, R. T. SMITH, P. BASTEN). Each group tries to conclude on models for lymphocyte activation in general. The situation is highly instructive and stimulating.

Volume 12 (12) on lymphocytes by allogeneic cells contains seven papers as well. The first two papers concentrate on genetic aspects of this model (WILSON *et al.*, F. H. RICH *et al.*). The predominance of the mixed lymphocyte reaction is elaborated then, the relation of *in vitro* sensitized T cells to the killer cell reaction (HARRY *et al.*). The other three papers discuss the relation *in vivo* by the graft versus host reaction on the one and tolerance (D. H. KATZ, P. MCCLELLAN) on the other. This volume combined with new data and attempts to render this volume a must for every immunologist.

T. L. VICTOR, Geneva

H. H. FELDBERG
New Oxford New

The inherited immunological mechanisms and molecules are the last years that the field has important influence has broadened and we have had a number of books. On 200 odd pages, the field including the new information to more general information extensively covered. After an intro-

P. SMITH and A. C. WARD, *Basic Immunogenetics*, London 1972 216 pp., £ 2.20

duction among individuals are often studied by differences are expressed on the immune lymphocytes. It became clear during the years of immunoglobulin and lymphocyte activity. Thus the field of immunogenetics that the current geneticists from San Francisco task is write a short new textbook, give not only an overall review of the field but they stimulate the reader and give and thought. Certain areas, of course, the personal work and experience of the authors are and evolution of immu-

lobulins are treated. This is followed by the genetics of the immunoglobulin molecules and the basis for antibody variability. The fifth chapter on cells, genes and the immune response reviews one of the most exciting and fast moving parts of immunogenetics. Finally, a long chapter is devoted to the human blood groups.

The book is easy to read, clear, stimulating and can be recommended to any body with interests in biology.

T. L. VISCHER, Geneva

Paroxysmal Nocturnal Hemoglobinuria (PNH) Series, Haematologica, vol. V/1
Munksgaard, Copenhagen 1972. 175 pp., dkr. 110.-

Dieser Band fasst in glanzender Weise alte und neueste Erkenntnisse dieses faszinierenden Krankheitsbildes zusammen. Er gibt eine gute Übersicht über die klinischen Verlaufsformen der PNH, Übergänge in aplastische Anämie, akute Leukämie und Myelofibrose. Diagnostische Tests werden *in extenso* besprochen. Biochemische und funktionelle Störungen der PNH-Zellen aller Linien werden in übersichtlicher Weise dargelegt. Alte und neue therapeutische Massnahmen werden kritisch analysiert. Dieses Übersichtswerk, an dem viele der grössten Kenner des Krankheitsbildes mitgearbeitet haben, kann jedem hämatologisch tätigen Arzt und jeder Bibliothek warmstens empfohlen werden.

H. SIECK, Basel

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T. L. VISCINA, Geneva

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B. SPICK, Basel

Studies on Bone Marrow Transplantation in Experimental ^{32}P -Induced Aplastic Anemia after Conditioning with Antilymphocyte Serum

H. STRECK and M. KISSLINO

Department of Hematology and the Unit for Protective Isolation
University Medical Center, Leiden

Abstract Aplastic anemia was produced with ^{32}P in rabbits with and without splenectomy. Transient hemopoietic engraftment without graft versus host (GVH) was seen after conditioning with antilymphocyte serum (ALS) and infusion of allogeneic marrow from a strain that is known to cause fatal secondary disease if total body irradiation is used for conditioning. More takes could be achieved in the splenectomized group but in both groups the takes were only transient. The absence of GVH was shown to be probably due to a split chimerism of the immunologic system. The graft rejections are felt to be due to the persistence of host T cells. Possibly the duration of engraftment can be prolonged in the future by continued immunosuppressive therapy after transplantation with ALS or cyclophosphamide. The fact that the spleen is not necessary for hemopoietic recovery after iv marrow infusion may have important clinical implications because many patients with aplastic anemia are splenectomized in the course of the disease.

Key Words
Antilymphocyte serum
Aplastic anemia
Bone marrow transplantation
Splenectomy

Despite modern therapeutic developments such as the use of anabolic steroids, transfusion of HL-A compatible platelets and granulocytes and nursing in a sterile environment, the prognosis of aplastic anemia (AA) is still very poor. Of 36 patients with this disease that we have seen on our Department of Hematology since 1967, two thirds have died and it has to be anticipated that even more of the patients with persisting pancytopenia in this series will have a fatal outcome.

It has been clearly demonstrated that bone marrow transplantation can be successful in AA after conditioning with cyclophosphamide (Cy) if

an HL-A identical sibling is available as marrow donor [1, 2, 3]. However, most of the patients are not in the fortunate situation of having an HL-A identical brother or sister. MATHE *et al* [4] were the first to show that takes can be achieved and graft versus host (GVH) avoided if anti-lymphocyte serum (ALS) alone is used for conditioning of these patients, even if the donor is not HL-A identical. We have confirmed this finding in rabbits with benzene induced aplastic anemia [5, 6]. The present study deals with ^{32}P induced marrow aplasia and bone marrow transplantation after conditioning with ALS.

Material and Methods

Marrow aplasia was induced in 40 male rabbits of a cross between Vienna White and Alaska of 1-2 kg. Group 1, consisting of 20 animals, was given 1.2 mCi/kg ^{32}P . Ten of these rabbits were given marrow grafts after conditioning with ALS. Five were transplanted without conditioning and 5 were not grafted. Group 2, consisting of 20 animals, was given 1.4 mCi/kg ^{32}P 10 days after they had been splenectomized. Ten of them were grafted after conditioning and 5 were not transplanted.

Conditioning was performed with 1 ml/kg ALS subcutaneously on 4 subsequent days. ALS was produced in a horse by repeated injections with rabbit thoracic duct lymphocytes. The efficacy of this serum was clearly established by prolongation of skin allograft survival and by *in vitro* inhibition of rosette formation [7]. Conditioning with ALS was started as soon as the peripheral white blood cell count had dropped below 1,500/mm³. This was usually 2-3 weeks after the ^{32}P . Marrow grafts were given 24h after the last dose of ALS. Female Dutch rabbits of 0.8-1.2 kg served as marrow donors. 2-3 $\times 10^6$ kg nucleated bone marrow cells were given intravenously. Previous marrow transplants between these 2 strains have been uniformly followed by fatal secondary disease if total body irradiation (TBI) was used for conditioning (SPRECKMEYER, BOORMAN and HOLLANDER, unpubl. data).

Follow-up studies. In all rabbits weekly blood counts, including hemoglobin, leukocytes, differential and platelets were performed. Cytogenetics of the bone marrow cells were performed as soon as there was any evidence of red cell chimerism. They were repeated if the chimerism lasted more than 8 weeks. A modified short term culture method of MOORHEAD [8, 9] was used. The proportion of XX and XY chromosomes were quantitated in at least 10 metaphases. Erythrocyte typing for the antigens A, B and F was performed every 1-2 weeks according to the method described by COHEN [10]. Drumsticks were looked for in the peripheral granulocytes once weekly. Immunoglobulin allotype studies were performed in the donor and recipient before grafting at 3 to 4 weekly intervals afterwards [11]. First and second set skin allograft survival studies from the previous marrow donor were performed on two long term chimeras. Two-way mixed leukocyte cultures between a long term chimera and the previous marrow donor were studied on day 50. Autopsies, histopathologic and bacteriologic studies were performed on all animals that died.

Table I. Course of control group not conditioned with ALS

	Number of animals	Died	Survival weeks	Cause of death		Hemopoietic recovery
				hemorrhage	sepsis	
No splenectomy 32P only	5	3	4 (2.5-5.5)	2	1	2
No splenectomy 32P plus marrow	5	5	3.8 (3-5)	3	2	-
Splenectomy 32P only	5	3	5.5 (3.5-6)	1	2	2
Splenectomy 32P plus marrow	5	3	5 (4-6)	-	3	2

14 animals died between 2.5 and 6 weeks. No evidence of donor marrow engraftment could be demonstrated in the 2 groups that were transplanted.

Results

Hematologic changes consisted mainly of thrombocytopenia and granulocytopenia which were noted in the second week after 32P. The absolute lymphocyte and hemoglobin counts decrease only minimally.

In the control group the 32P was not uniformly fatal, and 6 spontaneous hemopoietic recoveries occurred 4 times in animals that had 32P only and twice in animals that had a marrow graft. In the latter there was never any evidence of donor marrow engraftment. 14 control rabbits died between 2¹/₂ and 6 weeks after 32P either from septicemia or hemorrhage (table I).

Rabbits that were conditioned with ALS showed a high percentage of transient donor marrow engraftment (table II). Evidence of the take was seen between the third and fifth week after grafting. Karyotyping of the bone marrow cells usually showed between 20 and 40% of the cells to be of donor origin, but never more than 50%. Red cell typing with various dilutions of the antisera never indicated complete red cell chimerism. Drumsticks in the granulocytes were only occasionally found, but never reached the 2-3% that we had established to be normal for female rabbits of this strain. Immunoglobulin all type markers were present in 5 grafts. In 2 of them they turned to donor type after the third week and did not change any more during the follow-up period of over 10 weeks.

Table II Findings in the rabbits that were conditioned with ALS before bone marrow transplantation

	Number of animals	Evidence of take	Duration of take weeks	No take	Died too early for evaluation
1. Not splenectomized	10	4	7.5 (5-13)	2	4
2. Splenectomized	10	8	7 (5-12)	1	1

In splenectomized animals a much higher percentage of takes could be achieved, which may be due to the fact that in group 1, many animals died within a few days with severe pancytopenia. The duration of takes is comparable in both groups. In all the 12 animals that had transient takes of the donor marrow, their own hemopoiesis recovered and they became long term survivors.

First set skin allografts from the previous marrow donor were rejected between 10 and 12 days and second set rejections occurred within 7 days. The same first and second set rejection times were found in 4 normal controls of the same sex and strain. Also, the MLC between marrow donor and recipient was positive.

None of the animals developed any evidence of GVH. They behaved normally and gained weight normally. The animals dying during the course of the experiment were subjected to careful histological studies which did not yield any evidence of GVH.

Discussion

In ^{32}P induced aplastic anemia in rabbits a high percentage of takes of histo incompatible bone marrow can be achieved after conditioning with ALS. As in the previously reported rabbits with benzene induced aplastic anemia [5, 6], GVH is completely absent which in both experimental models is probably due to the fact that T cells remain of host type. The major difference between the two models was that the duration of marrow engraftment was much shorter in the ^{32}P induced aplasias. Here the first rejections occurred after 5 and the latest after 13 weeks. In the

benzene induced aplasias only one out of 6 animals that did have takes rejected the graft in the observation period of over 15 weeks.

The chimerism documented with cytogenetics of the bone marrow cells, red cell antigens and drumsticks, was much less complete in the ³²P than in the benzene induced aplasias. The chimerism of the immunologic system is comparable in both models. Unfortunately, immunoglobulin allotype markers were only present in 5 grafts. In 2 instances the allotypes turned to donor type after the third week and remained entirely donor during the follow up period of over 10 weeks. This implies a take of donor B cells. In the other 3 grafts there was either no take of B cells, or else the method was not sensitive enough to detect small amounts of donor immunoglobulin allotypes. The presence of host T cells during the chimeric state has been established by normal first and second set skin allograft rejections from previous marrow donor as well as with a positive MLC between donor and recipient.

It was very striking that animals that were previously splenectomized were much easier to transplant. Despite the fact that they had higher doses of ³²P, they developed less pronounced pancytopenia and also they tolerated the ALS better. This probably explains why more splenectomized animals survived the immediate post transplantation period. It is a well known fact of radiobiology that splenectomized animals tolerate higher doses of TBI [12] and chemotherapists know that splenectomized patients tolerate higher doses of cytostatic agents. The fact that hemopoietic recovery after intravenous bone marrow infusion can occur in rabbits without a spleen is very interesting and may have important clinical implications because many patients with AA are splenectomized in the course of their disease.

In 2 experimental models of AA, we have documented that after conditioning with ALS alone the host T cells are temporarily suppressed, which probably allows for a take of the donor marrow. Later the host T equivalent returns which may explain the absence of GVH and the frequent graft rejections. This has been confirmed in clinical patients with AA conditioned with ALS. GVH was absent and the take limited to erythropoiesis, myelopoiesis, thrombopoiesis and in several instances the B equivalent of the immunologic system [13-14]. Special emphasis has to be placed on the fact that all these takes were transient, lasting a maximum of 20 months [13]. The place of ALS for conditioning patients with AA for bone marrow transplantation has as yet to be definitely established. Great advantages are that fatal GVH can be avoided even if the do-

nor is not an HL-A identical sibling. However, the number of takes appears to be smaller than after conditioning with C₁ and only of transient nature [13]. On the other hand, they may suffice for spontaneous recovery of the patient's own hemopoietic system. In addition, new approaches for prevention of early graft rejection might be helpful possibly continued T cell suppression with ALS or C₁ after transplantation.

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Säulenchromatographische Anreicherung von DNA-Polymerase-Aktivitäten bei Leukämie

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Abstract Leukemic leukocytes revealed 2 species of DNA polymerases: DNA-dependent and RNA-dependent polymerase activities. The template characteristics of the RNA instructed enzymes were similar to those found with the DNA polymerase of oncogenic RNA viruses. Some minor differences in the molecular weight of RNA instructed DNA polymerase activities could be taken as arguments for some heterogeneity of reverse transcriptase in different forms of leukemia. The most striking difference of these results is to the DNA polymerase content of normal leukocytes or blast cells, inasmuch as normal blood cells lack RNA-dependent DNA polymerase activities. The possible role of reverse transcriptase in transforming cells led to the search for inhibitors. Therefore some rifamycins and distamycins have been tested for the influence on DNA instructed DNA synthesis in a cell free system.

Key Words

Chromatography
DNA polymerases
Leukemic cells
Rifamycins

Nach bisher vorliegenden Literaturangaben erscheint es, dass die Umkehr des Flusses der genetischen Information, also die Übertragung von RNA auf DNA durch das Enzym reverse Transkriptase in RNA-Viren spezifisch für Onkogenität [6] und in eukaryoten Zellen nur in embryonalen oder Tumorzellen aufgefunden werden kann [1, 3-8].

Die Ätiologie der menschlichen Leukämie ist bisher unbekannt. Aus den verschiedensten Überlegungen wird jedoch ein onkogenes RNA-Virus als mögliche Ursache dieser Erkrankung diskutiert [4-8]. Die Suche nach RNA-Viruspartikeln in Leukämiezellen ist bisher jedoch durchwegs negativ verlaufen. Da offensichtlich der Virusnachweis mit den heutigen Methoden noch nicht gelingt, könnte der Nachweis eines

viralen Enzyms mit den chemischen Eigenschaften des Ternin-Baltimore-Enzyms [2, 17] hierfür eine sensitivere Nachweismethode darstellen

Material und Methoden

2 - 14 C-Thymidintriphosphat (spez. Akt. 32 mCi/mm) wurde vom Radiochemical Centre Amersham bezogen. Poly rAdT, Poly d (AT), rAdT₁₀ und dAdT₁₀ von Miles, DEAE Zellulose G5, Zytocrom C, MG 12400 Chymotrypsinogen A, MG 23 000, Rinderaktin, MG 67 000 und Aldofase, MG 160 000, von Serva, Phosphozellulose von Whatman, Noradren von Shell, dATP, dCTP, dGTP, Dithiothreitol und Sephadex G 200 von Sigma. Das Distamycinderivat Nr. 18230 wurde uns von der Firma Farmitalia zur Verfügung gestellt. Die Rifamycinderivate 49623 Ba, 49731 Ba und 18414 Ba wurden uns von der Ciba-Geigy überlassen. Alle übrigen Reagentien waren P.a.-Qualität und stammten von Sigma oder Merck.

Die Blutzellen wurden von Patienten mit akuter Leukämie (AL, POX Typ) chronisch myelischer Leukämie (CMV) und chronisch lymphatischer Leukämie (CLL) gewonnen. Bei allen Spendern wurde die klinische Diagnose durch Sternalpunktion und histochemische Spezialfärbungen gesichert. Die für unsere Untersuchungen herangezogenen Patienten waren entweder unbehandelt oder hatten während der letzten 3 Monate vor der Entnahme von Blutzellen keine Zytostatika oder Glukokortikoide erhalten. Die Gewinnung der Blutzellen erfolgt mit der Zellsufuge von Aminen. Die entnommenen Zellzahlen schwankten zwischen 10^6 und $8 \cdot 10^{11}$ Leukozyten.

1 Präparation der DNA Polymerasen aus leukämischen Leukozyten Die unter sterilen Kautelen gewonnenen Leukämiezellen wurden 10 min bei 60 g zentrifugiert und das überschüssige Plasma abgewaschen. Das resultierende Zellpellet wurde in 2 vol Phosphatpuffer (0,01 M pH 7,2, 20% Glycerin, 0,001 M EDTA, 0,001 M DTT, 0,1% Nadeosylat, 7% Noradren) suspendiert und im Braun Mixer homogenisiert. Das Homogenat wurde 20 min bei 6000 g und der resultierende Überstand bei 105 000 g 1 h zentrifugiert.

2 Anreicherung der DNA Polymerasen auf DEAE Zellulose Puffer A: 0,05 M Phosphatpuffer pH 7,2, 2 mM DTT, 10% Glycerin. Puffer B: 0,3 M Phosphatpuffer pH 7,2, 2 mM DTT, 10% Glycerin. Puffer C: 2 mM DTT, 10% Glycerin.

200 g DEAE Zellulose wurden auf einem Büchner Trichter mit 3 l Puffer A befeuchtet. Anschließend wurde der 105 000-g-Überstand 10 min mit Puffer B verdünnt und auf die Zellulose aufgetragen, mit 5 l Puffer A nachgewaschen und eine Proteinfraktion mit 3 l Puffer B eluiert. Die Washlösung wurde 3 mal die Filtration flach mit Puffer C verdünnt. Das pH der beiden Lösungen wurde mit KOH auf 8,0 eingestellt.

3 Weitere Anreicherung von DNA Polymerasen durch Chromatographie auf Phosphorcellulose Puffer A: 0,01 M Phosphatpuffer pH 8,0, 2 mM DTT, 10% Glycerin. Puffer B: 0,01 M Phosphatpuffer pH 8,0, 2 mM DTT, 10% Glycerin.

Zwei 4 cm messende Säulen wurden 1 m hoch mit Phosphozellulose gefüllt und mit 2 vol Puffer A befeuchtet. Auf eine Säule wurden die unter Punkt 2 erhaltene Washlösung und auf die andere Säule die Filtration aufgetragen. Die

Elution der DNA-Polymerasen erfolgte mit einem linearen Phosphatpuffergradient (je 2 l Puffer A Puffer B) und mit Hilfe einer peristaltischen Pumpe. Die Flussrate betrug etwa 0,7 ml/min. Es wurden Fraktionen zu ungefähr 20 ml gesammelt und auf Polymeraseaktivität getestet. Die Gipfelfraktionen wurden gepoolt und im Amicon Ultrafiltriergerät konzentriert (PM 10 Filter).

4 *Bestimmung des Molekulargewichtes durch Gel-Filtration auf Sephadex G 200* 75 g Sephadex G 200 wurden 3 Tage in 0,15 M Phosphatpuffer (pH 8,0) 2 mM DTT und 10% Glycerin quellen gelassen, und anschließend wurde eine 5 cm im Durchmesser messende Säule 1,3 m hoch gefüllt. Nach Äquilibrieren mit 2 Molen volumina Phosphatpuffer wurden die Enzymlösungen aufgetragen. Es wurden Fraktionen zu etwa 20 ml gesammelt. Als Referenzverbindungen wurden Dextran blau, Zytosin C, MG 12 400 Chymotrypsinogen, MG 25 000 Rinderalbumin, MG 67 000 und Aldolase, MG 160 000 analog chromatographiert.

5 *Bestimmung der Polymeraseaktivität* In 100 µl Testansatz waren enthalten: 40 mM Tris-HCl pH 7,8, 80 mM KCl, 10 mM MgCl₂, 5 mM DTT, 0,08 mM TTP, 15 µl TTP-¹⁴C, je 0,08 mM dATP, dCTP, dGTP, 10 µg Rinderalbumin und 0,5 µg Template-DNA. Zu der angegebenen Mischung wurden 20 µl Enzymlösung zugeetzt und das Inkubationsgemisch 30 min bei 37 °C inkubiert. Nach Abschluss der Inkubationsperiode wurden 100 µl Anteile auf Whatman 3MM Filterpapierchen aufgetragen, die Plättchen getrocknet und auf einem Büchner-Trichter in der Reihenfolge mit den folgenden Lösungen je 2% gewaschen (zirka 200 ml): 1% Trichloressigsäure (TCA), 5% TCA + 1% Tetranatriumpyrosulfat, 5% TCA, Äthanol, Äther. Nach dem Trocknen wurden die Plättchen mit 10 ml Toluolszintillator (6 g Butyl-PBD in Toluol) im Tri-Carb (Packard) gezählt. Alle Ergebnisse wurden auf einen mit diesem Versuchansatz ermittelten Blindwert korrigiert.

6 *Einflussung der ¹⁴C-TTP-Inkorporation durch Zusatz von Distamycin-derivaten bzw. Rifamycin-derivaten in den Testansatz zur Bestimmung der Polymeraseaktivität* Die Distamycin- oder Rifamycin-derivate wurden in Dimethylsulfoxyd (DMSO) gelöst und dem unter Punkt 5 beschriebenen Testansatz zugegeben. Blindwert und Leerwert erhielten lediglich den Zusatz einer adäquaten Menge DMSO.

Ergebnisse

Das Elutionsprofil der DNA-abhängigen DNA-Polymerasen wurde durch Austesten der einzelnen Fraktionen mit d(A-T) oder d(A-T)_n ermittelt, das der RNA-abhängigen DNA-Polymerasen mit den Templates r(A-T) bzw. r(A-T)_n [6, 7, 9, 14, 16].

1 *Chromatographisches Verhalten der DNA-Polymerasen aus Leukämischen Zellen* Die an DEAE-Zellulose (0,05–0,3 M Phosphatpuffer) vorgereinigten Enzyme wurden auf Phosphozellulose aufgetragen und mit Hilfe eines Phosphatpuffergradienten eluiert. Die einzelnen Fraktionen wurden gesammelt und auf Polymeraseaktivität getestet. Diese Enzympreparationen waren offensichtlich weitgehend frei von

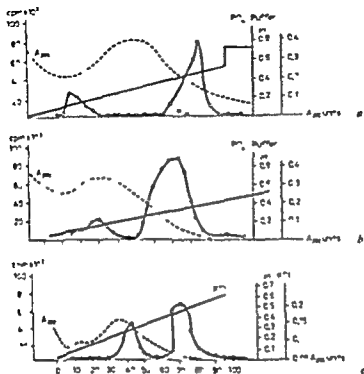


Abb. 1 Chromatographisches Verhalten der DNA Polymerasen menschlicher Leukämiezellen nach Verteilung an DEAE Zellulose (0,05–0,3 M Phosphatpuffer) und anschließender Chromatographie auf Phosphorellulose. Die Polymerasen wurden von der Phosphorellulose durch Anlegen eines linearen Phosphatpuffergradienten eluiert. Es wurden Fraktionen zu etwa 20 ml gesammelt und Aliquote von 20 µl jeweils auf Polymeraseaktivität mit d(AT) als Template angewendet. Ordinate: cpm/20 µl Fraktion. Fluoreszenzprofile der D-DNA Polymerasen bei CLL (a), CMV (b) und AL, POX Typ (c).

Nukleinsäuren mit der D-Phenylisourzinnation konnte kein DNA-Gehalt nachgewiesen werden. Die Abbildungen 1a und 1b zeigen das Elutionsprofil der D-DNA Polymerasen, die von Patienten mit CLL bzw. CMV gewonnen wurden. Die beiden Chromatogramme erscheinen nahezu identisch. Abbildung 1c stellt das chromatographische Verhalten der D-DNA Polymerasen bei AL dar. Es zeigt ebenfalls 2 Peaks, durch ihrer Fluoreszenz von Phosphorellulose von Gallen als Polymera-

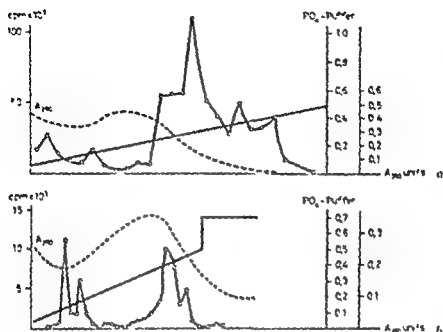


Abb. 2 Chromatographisches Verhalten der R-DNA-Polymerase Aktivitäten aus menschlichen Leukämiezellen nach Vorreinigung an DiAT-Zellulose (0.05–0.3 M Phosphatpuffer) und anschließender Chromatographie auf Phosphozellulose. Die Polymerasen wurden von der Phosphozellulose durch Anlegen eines linearen Phosphatpuffergradienten eluiert. Es wurden Fraktionen zu zirka 20 ml gesammelt und Anteile von 20 μl mit rAdT als Template auf DNA-Polymerase Aktivität getestet. Ordinate: $\text{cpm}/20 \mu\text{l}$ Enzym. Abszisse: Fraktionsnummern. Fluoroprofile der \blacksquare DNA-Polymerasen bei CLL (a) und CML (b).

se I und Polymerase II bezeichnet werden. Gegenüber den Abbildungen 1a und 1b fällt auf, dass die Polymerase I geringfügig höher ist und erst mit hohermolarer Salzkonzentration eluiert werden kann.

2. *Chromatographisches Verhalten der R-DNA-Polymerase-Aktivitäten aus leukämischen Zellen.* Abbildung 2a zeigt das chromatographische Verhalten der R-DNA-Polymerase-Aktivitäten aus CML, Abbildung 2b bei CLL. Ähnliche Profile wurden auch bei AL erhalten. Gemeinsam war allen 3 Leukämieformen, dass sich mit den Templates rAdT und rAdT₁₀ durchwegs eine mehrgipfelige Kurve ergab. Man könnte fälschlicherweise vermuten, dass es sehr viele verschiedene reverse Transkriptasen in Leukämiezellen geben könnte. Um diese Frage näher abzuklären, wurden alle Gipfelfraktionen einer Sephadexchroma-

Tabelle 1 Beeinflussung der ^{14}C -TTP Inkorporation durch Zugabe von Distamycin-derivaten (Substanz 182 = CXIII) bzw. Rifamycinderivaten (Substanz 45/23-Ba = C 11, 45/14-Ba = C 22, 45/33 Ba = C 27) in den Testansatz zur Bestimmung der Enzymaktivität der Polymerasen I und II aus leukämischen Leukocyten

Substanz	Menge µg	Polymerase I %	Polymerase II %
Kompletter Ansatz		100	100
C XIII	5	36	84
	10	14	34
C 11	5	96	102
	10	270	190
	15	930	820
C 22	5	87	93
	10	360	270
	15	860	1002
C 27	5	113	107
	10	470	240
	15	890	810

topographie unterworfen und die mit dieser Chromatographie erhaltenen Peaks auf Templatecharakteristika untersucht. Wie in Tabelle II gezeigt wird, erfüllt jedoch lediglich ein Gipfel alle Voraussetzungen für eine reverse Transkriptase. Die Ursache für diese vielgipfligen Kurven liegt also lediglich darin, dass uns kein »spezifisches« Template für die reverse Transkriptase zur Verfügung stand und aus diesem Grunde vielschichtigere Parameter für den Enzymnachweis berücksichtigt werden mussten [3-8, 14-16].

3. Beeinflussung der D-DNA Polymerasen I und II durch Zusatz von Rifamycin- und Distamycinderivaten. Für diese Untersuchungen wurden Substanzen gewählt, welche bekanntlich die RNA-abhängige DNA-Polymerase onkogener RNA-Viren hemmen [8, 9-10]. Während Rifamycin-derivate eher eine Aktivierung der D-DNA Polymerasen leukämischer Zellen herbeiführen, bewirkte Distamycin eine Hemmung der beiden D-DNA Polymerasen (Tab. I). Die Hemmung der untersuchten Substanzen auf die R-DNA-Polymerase konnte bestätigt werden.

4. Mischungsverhältnisse von D-DNA- und R-DNA-Polymerasen auf Sephadex G-200. Die Peakfraktionen der Phosphoräzidechromatographie wurden jeweils auf einer Sephadex-G-200-Säule aufgetragen. Das Ergebnis, welches für die template-lose Leukämie erhalten

Tabelle II Template-(Primer) Charakteristika von DNA Polymerase-Aktiv I en bei Leukämie (TTP Inkorporation in pMol/h µg Protein). Die in der Sephadexchromatographie bestimmten Fraktionen Polymerase I, Polymerase II und reverse Transkriptase wurden gesammelt, eingereigt und mit den verschiedenen Templates auf DNA Polymerase-Aktivität getestet

Template			DNA Polymerase		
			I	II	Reverse
dT ₁₀ rA	Mg ⁺⁺		12	0	361,00
dT ₁₀ rA	Mn ⁺⁺ (Mg)		23	10	422,00
dT ₁₀ dA	Mg ⁺⁺		97	5400	320,00
Poly rA dT	Mg ⁺⁺		0	20	94,00
Poly rA dT	Mn ⁺⁺ (Mg)		56	40	125,00
Poly dA dT	Mg ⁺⁺		44	2570	38,30
DNA nativ	Mg ⁺⁺		2	670	13,00
DNA denat	Mg ⁺⁺		3	2060	24,00
Poly rA rU	Mg ⁺⁺		3	0	290,00
Poly rA rU	Mn ⁺⁺ (Mg)		9	0	321,00
tRNA ^{Pro} _{Leu}	Mg ⁺⁺		12	120	354,00
Q ₈ RNA + dT					
¹⁴ C TTP	Mg ⁺⁺		10	40	323,00
Q ₈ RNA + dT					
³ H-dGTP	Mg ⁺⁺		0	0	287,00

wurde, ist in Abbildung 3, das der myeloischen Leukämie in Abbildung 4 wiedergegeben. Bei beiden Leukämieformen konnten 2 D DNA-Polymerasen nachgewiesen werden, welche analoges Verhalten hinsichtlich ihres Molekulargewichtes aufweisen. Die Gipfel der R-DNA-Polymerasen verhalten sich jedoch bei den von uns untersuchten Fällen verschieden. So besitzen die R-DNA-Polymerase-Aktivitäten aus myeloischer Leukämie ein wesentlich höheres Molekulargewicht als bei lymphatischer Leukämie. Besonders erachtenswert erscheint es dabei, dass die Chromatographie der R-DNA-Polymerasen eine Reihe von Fraktionen sowohl bei myeloischer wie bei lymphatischer Leukämie ergab, welche rA.dT₁₀ weit besser als dA.dT₁₀ ablesen können.

Das Molekulargewicht der DNA-Polymerase I beträgt etwa 150 000, das der Polymerase II etwa 30 000. Die Template-(Primer) Charakteristika werden in der Tabelle II wiedergegeben. Diese Untersuchungen wurden mit den Lezytpräparationen der Sephadexchromatographie durchgeführt.

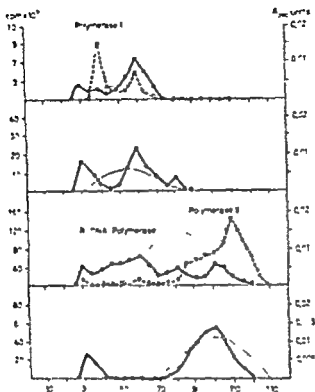


Abb. 3. Chromatographisches Verhalten der D-DNA-Polymerasen aus Abbildung 1a und der R-DNA-Polymerasen aus Abbildung 2a auf Sephadex G 200. Das Elutionsprofil der Polymerasen der Phosphorcellulosechromatographie wurde den Fraktionen entsprechend in 4 Teile eingeteilt. Diese Gelfraktionen wurden gesammelt und durch Ultrafiltration auf ein Volumen von etwa 10 ml eingedampft (Amicon, PM 15-Filtrier). Die 4 Chromatographiesäulen wurden in der entsprechenden Reihenfolge auf eine 1,2 m Höhe und 3 cm im Durchmesser stehende Chromatographiesäule aufgetragen und jeweils eluiert. Die Kurven stellen das Elutionsverhalten dieser Polymerasen in einem noch Gefrierfahigen Gel dar. Es wurden Fraktionen zu je 20 ml gesammelt und 20 μ l A₂₆₀ auf Polymeraseaktivität des Template dAdT₁₀ (●) und dAdT₁₀ (○) getestet. Ordinate: je 20 μ l Fraktion Ablesung Fraktionennummer. — A₂₆₀ mit 0,1.

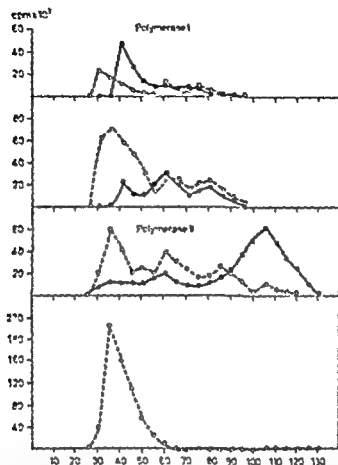


Abb 4 Chromatographisches Verhalten der Peakfraktionen der D DNA Polymerasen aus Abbildung 1b und der R DNA Polymerasen aus Abbildung 2b auf Sephadex G 200. Versuchsordnung: siehe Legende zur Abbildung 3. ○ = R DNA Polymerase Aktivität, ● = D DNA Polymerase Aktivität.

Diskussion

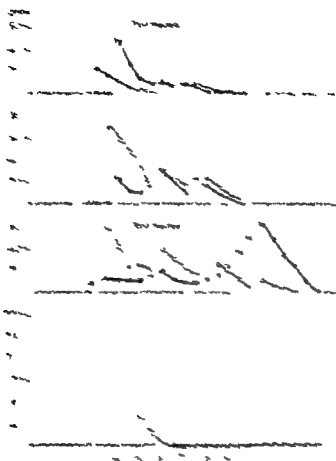
In unseren Untersuchungen wurden DNA-Polymerase-Aktivitäten chromatographisch hoher angereichert und fraktioniert. Die Profile der D DNA- und R-DNA-Polymerase Aktivitäten bei AL, CLL und CML waren in unserer Versuchsanordnung nicht wesentlich unterschieden. Die Ergebnisse stehen im Einklang mit den Befunden von GALLO [6], GALLO *et al* [5, 7, 8] und LOEB *et al* [15], obwohl wir bestrebt waren durch Behandlung der intakten Zellen mit sehr hohen Detergensen-

trationen sämtliche in Leukämiezellen vorkommende Polymerasen zu extrahieren. Allerdings fanden wir im Gegensatz zu GALLO bei CML und CLL RNA Polymerase Aktivitäten welche nach chromatographischen Kriterien verschiedene Enzyme sein könnten.

Ein bemerkenswerter Unterschied gegenüber normalen Blutzellen oder nicht leukämischen Blasten liegt jedoch darin, dass in leukämischen Zellen DNA Polymerase Aktivitäten beobachtet werden konnten welche rAdT_{10} weit besser ableasen können als dAdT_{10} [1, 3-8, 14]. Wie wir nachweisen konnten, ist diese RNA Polymerase auch in der Lage das Template Q-RNA abzulesen. Die nachgewiesene Inkorporation in das durchfallbare Präzipitat mit $^{14}\text{C-TTP}$ und $^3\text{H-dGTP}$ spricht in diesem Zusammenhang dafür, dass wirklich heteropolymere Anteile einer Ribonukleinsäure in eine Desoxyribonukleinsäure transkribiert wurden. Da die Q-RNA die Ribonukleinsäure eines RNA Virus darstellt haben wir in unserem Versuch demnach ein DNA Provirus eines nicht onkogenen RNA Virus im zellfreien System hergestellt.

Die Transkription heteropolymerer RNA Anteile und die Bevorzugung des Templates rAdT_{10} über dAdT_{10} ist nach GALLO *et al.* [8] für das Vorliegen einer reversen Transkriptase charakteristisch. Der grosse methodische Aufwand des Nachweises der reversen Transkriptase in leukämischen Blutzellen hat es uns leider nicht ermöglicht, eine grosse Anzahl von Leukämiepatienten systematisch auf das Vorkommen dieses Enzyms zu untersuchen. Die vorliegenden Literaturangaben könnten jedoch im Zusammenhang mit unseren Ergebnissen dafür sprechen, dass Leukämiezellen von normalen Blutzellen durch das Vorkommen dieses Enzyms unterschieden werden könnten. Der Nachweis einer RNA abhängigen DNA Polymerase in Leukämiezellen im Gegensatz zu normalen Blutzellen wäre vereinbar mit den molekularen Hybridierungsuntersuchungen und den vergleichenden säulenchromatographischen Untersuchungen [4, 12] welche für eine virale Genese der menschlichen Leukämie sprechen. Es erscheint dabei, dass das Virus RNA-Genom in die Zelle integriert und nur noch funktionell erfassbar wurde (DNA Provirus). Die Information der von RNA auf DNA übertragen wird wirft die interessante Frage der «Gene Amplifikation» (Tsuchida, Valenzuela) während der klonematischen Zellvermehrung auf. Da die Leukämie auch als Störung der Zellteilung und -differenzierung angesehen wird [4, 5] können sich also aus neuen Aspekten der Leukämiegenese ergeben.

Die bisherige Behandlung der Leukämie mit ganz unspezifisch wirkenden Substanzen der Zellstörungsbehandlung mag es erdem Endziel



1. 2. 3. 4. 5. 6. 7. 8. 9. 10. 11. 12. 13. 14. 15. 16. 17. 18. 19. 20. 21. 22. 23. 24. 25. 26. 27. 28. 29. 30. 31. 32. 33. 34. 35. 36. 37. 38. 39. 40. 41. 42. 43. 44. 45. 46. 47. 48. 49. 50. 51. 52. 53. 54. 55. 56. 57. 58. 59. 60. 61. 62. 63. 64. 65. 66. 67. 68. 69. 70. 71. 72. 73. 74. 75. 76. 77. 78. 79. 80. 81. 82. 83. 84. 85. 86. 87. 88. 89. 90. 91. 92. 93. 94. 95. 96. 97. 98. 99. 100. 101. 102. 103. 104. 105. 106. 107. 108. 109. 110. 111. 112. 113. 114. 115. 116. 117. 118. 119. 120. 121. 122. 123. 124. 125. 126. 127. 128. 129. 130. 131. 132. 133. 134. 135. 136. 137. 138. 139. 140. 141. 142. 143. 144. 145. 146. 147. 148. 149. 150. 151. 152. 153. 154. 155. 156. 157. 158. 159. 160. 161. 162. 163. 164. 165. 166. 167. 168. 169. 170. 171. 172. 173. 174. 175. 176. 177. 178. 179. 180. 181. 182. 183. 184. 185. 186. 187. 188. 189. 190. 191. 192. 193. 194. 195. 196. 197. 198. 199. 200. 201. 202. 203. 204. 205. 206. 207. 208. 209. 210. 211. 212. 213. 214. 215. 216. 217. 218. 219. 220. 221. 222. 223. 224. 225. 226. 227. 228. 229. 230. 231. 232. 233. 234. 235. 236. 237. 238. 239. 240. 241. 242. 243. 244. 245. 246. 247. 248. 249. 250. 251. 252. 253. 254. 255. 256. 257. 258. 259. 260. 261. 262. 263. 264. 265. 266. 267. 268. 269. 270. 271. 272. 273. 274. 275. 276. 277. 278. 279. 280. 281. 282. 283. 284. 285. 286. 287. 288. 289. 290. 291. 292. 293. 294. 295. 296. 297. 298. 299. 300. 301. 302. 303. 304. 305. 306. 307. 308. 309. 310. 311. 312. 313. 314. 315. 316. 317. 318. 319. 320. 321. 322. 323. 324. 325. 326. 327. 328. 329. 330. 331. 332. 333. 334. 335. 336. 337. 338. 339. 340. 341. 342. 343. 344. 345. 346. 347. 348. 349. 350. 351. 352. 353. 354. 355. 356. 357. 358. 359. 360. 361. 362. 363. 364. 365. 366. 367. 368. 369. 370. 371. 372. 373. 374. 375. 376. 377. 378. 379. 380. 381. 382. 383. 384. 385. 386. 387. 388. 389. 390. 391. 392. 393. 394. 395. 396. 397. 398. 399. 400. 401. 402. 403. 404. 405. 406. 407. 408. 409. 410. 411. 412. 413. 414. 415. 416. 417. 418. 419. 420. 421. 422. 423. 424. 425. 426. 427. 428. 429. 430. 431. 432. 433. 434. 435. 436. 437. 438. 439. 440. 441. 442. 443. 444. 445. 446. 447. 448. 449. 450. 451. 452. 453. 454. 455. 456. 457. 458. 459. 460. 461. 462. 463. 464. 465. 466. 467. 468. 469. 470. 471. 472. 473. 474. 475. 476. 477. 478. 479. 480. 481. 482. 483. 484. 485. 486. 487. 488. 489. 490. 491. 492. 493. 494. 495. 496. 497. 498. 499. 500. 501. 502. 503. 504. 505. 506. 507. 508. 509. 510. 511. 512. 513. 514. 515. 516. 517. 518. 519. 520. 521. 522. 523. 524. 525. 526. 527. 528. 529. 530. 531. 532. 533. 534. 535. 536. 537. 538. 539. 540. 541. 542. 543. 544. 545. 546. 547. 548. 549. 550. 551. 552. 553. 554. 555. 556. 557. 558. 559. 560. 561. 562. 563. 564. 565. 566. 567. 568. 569. 570. 571. 572. 573. 574. 575. 576. 577. 578. 579. 580. 581. 582. 583. 584. 585. 586. 587. 588. 589. 590. 591. 592. 593. 594. 595. 596. 597. 598. 599. 600. 601. 602. 603. 604. 605. 606. 607. 608. 609. 610. 611. 612. 613. 614. 615. 616. 617. 618. 619. 620. 621. 622. 623. 624. 625. 626. 627. 628. 629. 630. 631. 632. 633. 634. 635. 636. 637. 638. 639. 640. 641. 642. 643. 644. 645. 646. 647. 648. 649. 650. 651. 652. 653. 654. 655. 656. 657. 658. 659. 660. 661. 662. 663. 664. 665. 666. 667. 668. 669. 670. 671. 672. 673. 674. 675. 676. 677. 678. 679. 680. 681. 682. 683. 684. 685. 686. 687. 688. 689. 690. 691. 692. 693. 694. 695. 696. 697. 698. 699. 700. 701. 702. 703. 704. 705. 706. 707. 708. 709. 710. 711. 712. 713. 714. 715. 716. 717. 718. 719. 720. 721. 722. 723. 724. 725. 726. 727. 728. 729. 730. 731. 732. 733. 734. 735. 736. 737. 738. 739. 740. 741. 742. 743. 744. 745. 746. 747. 748. 749. 750. 751. 752. 753. 754. 755. 756. 757. 758. 759. 760. 761. 762. 763. 764. 765. 766. 767. 768. 769. 770. 771. 772. 773. 774. 775. 776. 777. 778. 779. 780. 781. 782. 783. 784. 785. 786. 787. 788. 789. 790. 791. 792. 793. 794. 795. 796. 797. 798. 799. 800. 801. 802. 803. 804. 805. 806. 807. 808. 809. 810. 811. 812. 813. 814. 815. 816. 817. 818. 819. 820. 821. 822. 823. 824. 825. 826. 827. 828. 829. 830. 831. 832. 833. 834. 835. 836. 837. 838. 839. 840.

22

The purpose of this study is to determine the effect of the use of the
 computer on the learning of the English language. The study was conducted
 in a classroom where the students were taught English. The results of the
 study show that the use of the computer has a positive effect on the
 learning of the English language. The students who used the computer
 showed a higher level of achievement than the students who did not use
 the computer. The study also found that the use of the computer
 increased the students' motivation to learn English. The students who
 used the computer were more interested in the subject and spent more
 time on their homework. The study concludes that the use of the
 computer is an effective way to teach the English language.

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Ein bemerkenswerter Unterschied gegenüber normalen Blutzellen oder nicht leukämischen Blasten liegt jedoch darin, dass in leukämischen Zellen DNA-Polymerase-Aktivitäten beobachtet werden konnten, welche rAdT_{12} weit besser ablesen können als dAdT_{12} [1, 3-8, 14]. Wie wir nachweisen konnten, ist diese R-DNA-Polymerase auch in der Lage, das Template Q β -RNA abzulesen. Die nachgewiesene Inkorporation in das säurefällbare Präzipitat mit ^{14}C -TTP und ^3H dGTP spricht in diesem Zusammenhang dafür, dass wirklich heteropolymere Anteile einer Ribonukleinsäure in eine Desoxyribonukleinsäure transkribiert wurden. Da die Q β -RNA die Ribonukleinsäure eines RNA-Virus darstellt, haben wir in unserem Versuch demnach ein DNA-Provirus eines nicht onkogenen RNA-Virus im zellfreien System hergestellt.

Die Transkription heteropolymere RNA-Anteile und die Bevorzugung des Templates rAdT_{12} über dAdT_{12} ist nach Gallo *et al.* [8] für das Vorliegen einer reversen Transkriptase charakteristisch. Der grosse methodische Aufwand des Nachweises der reversen Transkriptase in leukämischen Blutzellen hat es uns leider nicht ermöglicht, eine grosse Anzahl von Leukamiepatienten systematisch auf das Vorkommen dieses Enzyms zu untersuchen. Die vorliegenden Literaturangaben könnten jedoch im Zusammenhang mit unseren Ergebnissen dafür sprechen, dass Leukamiezellen von normalen Blutzellen durch das Vorkommen dieses Enzyms unterschieden werden könnten. Der Nachweis einer RNA-abhängigen DNA-Polymerase in Leukamiezellen im Gegensatz zu normalen Blutzellen wäre vereinbar mit den molekularen Hybridisierungsuntersuchungen und den vergleichenden säulenchromatographischen Untersuchungen [4, 12] welche für eine virale Genese der menschlichen Leukämie sprechen. Es erscheint dabei, dass das Virus RNA-Genom in die Zelle integriert und nur noch funktionell erfassbar würde (DNA Provirus). Die Information die von RNA auf DNA übertragen wird, wirft die interessante Frage der «Gene Amplifikation» (Tocchioni-Valentini) während der tumorentstehenden Zellproliferation auf. Da die Leukämie auch als Störung der Zellteilung und -differenzierung angesehen wird [4, 9] könnten sich daraus neue Aspekte in der Leukämiegenese ergeben.

Die bisherige Behandlung der Leukämie mit ganz empfindlich wirkenden Substanzen der Zellwachstumshemmung mag unter dem Eindruck

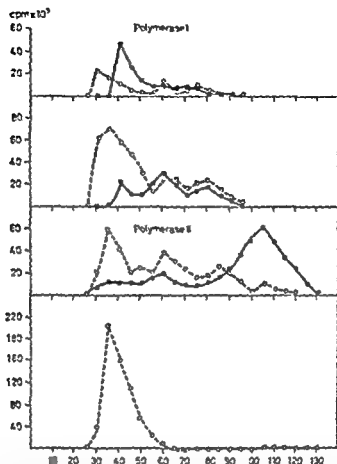


Abb 4 Chromatographisches Verhalten der Peakfraktionen der D-DNA Polymerasen aus Abbildung 1b und der R-DNA Polymerasen aus Abbildung 2b auf Sephadex G-200. Versuchsordnung siehe Legende zur Abbildung 3 ○ = R-DNA Polymerase Aktivität, ● = D-DNA Polymerase Aktivität

Diskussion

In unseren Untersuchungen wurden DNA-Polymerase-Aktivitäten chromatographisch hoher angereichert und fraktioniert. Die Profile der D-DNA- und R-DNA-Polymerase-Aktivitäten bei AL, CLL und CML waren in unserer Versuchsanordnung nicht wesentlich unterschieden. Die Ergebnisse stehen im Einklang mit den Befunden von GALLO [6], GALLO *et al* [5, 7, 8] und LOEB *et al* [15], obwohl wir bestrebt waren, durch Behandlung der intakten Zellen mit sehr hohen Detergenskonzentrationen

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Die bisherige Behandlung der Leukämie mit ganz unspezifisch wirkenden Substanzen der Zellteilungshemmung mag unter dem Eindruck

der sich mehrenden Berichte über eine Störung der Zelldifferenzierung bei diesem Krankheitsbild als nicht therapeutisch zum Ziele führend erscheinen. Die Isolierung RNA-abhängiger DNA-Polymerasen aus Blutzellen [5, 6, 10] und onkogenen RNA-Viren wurde daher als *In-vitro*-Ansatz zur Auffindung antileukämischer Substanzen herangezogen. In der Zwischenzeit wurden verschiedenste Verbindungen ermittelt, welche die DNA-Polymerase der RNA-Viren hemmen [6, 10, 11]. In vielen Fällen wurde jedoch dabei außer Betracht gelassen, dass diese Hemmstoffe möglichst spezifisch auf das virale Enzym wirken sollten, die normalen und zellständigen eukaryoten DNA-Polymerasen jedoch möglichst unbeeinflusst bleiben sollten. Die von uns untersuchten Rifamycinderivate scheinen dafür gewisse Voraussetzungen zu erfüllen und berechtigen zu weiteren Untersuchungen. Das Distamycinderivat mit der beobachteten Hemmwirkung auf DNA-abhängige DNA-Polymerasen erscheint dagegen als ein weit weniger spezifischer Hemmstoff. In diesem Zusammenhang ist es von Interesse, dass von verschiedenen Laboratorien über eine selektive Toxizität von Hemmstoffen der reversen Transkriptase auf transformierte Zellen berichtet wurde [6, 11].

Das Studium der DNA-Polymerasen normaler und leukämischer Zellen beansprucht daher in besonderer Weise experimentelles Interesse, um die pathophysiologischen Grundzüge der Leukämie besser verstehen zu können. Das Konzept, dass die in Leukämiezellen vorkommende reverse Transkriptase eine Rolle für das Initialstadium der Leukämie spielt und unter Umständen auch daran beteiligt ist, das Stadium der Malignität aufrechtzuerhalten, liesse daran denken, dass die Anwendung von Hemmstoffen der RNA-abhängigen DNA-Polymerasen ein neues therapeutisches Prinzip für die Behandlung maligner Erkrankungen darstellen könnte.

Diese Untersuchungen wurden mit Unterstützung des Österreichischen Forschungsrates (Projekt 1477) und im Auftrag des österreichischen Bundesministeriums für Wissenschaft und Forschung durchgeführt.

Zusammenfassung

In leukämischen Leukozyten konnten 2 verschiedene DNA Polymerase Aktivitäten durch säulenchromatographische Auftrennung nachgewiesen werden. DNA abhängige DNA Polymerasen (Polymerase I und Polymerase II) und RNA abhängige Polymeraseaktivitäten. Die Template (Primer) Charakteristika dieser RNA

abhängigen DNA Polymerasen waren ähnlich wie für die reverse Transkriptase onkogener RNA Viren. Gewisse Unterschiede im Molekulargewicht RNA abhängiger DNA Polymerasen bei lymphatischer Leukämie gegenüber myeloischer Leukämie sprechen für eine gewisse Heterogenität dieser Enzyme bei verschiedenen Leukämiespezies. Wesentlich ist der Unterschied zu normalen Blutzellen oder nicht leukämischen Blasten, da in diesen Zellen bisher niemals eine reverse Transkriptase nachgewiesen werden konnte. Die mögliche Beteiligung dieses Enzyms bei den Vorgängen der Malignität hat uns daher veranlaßt, in einem *In vitro*-screening System verschiedene Hemmstoffe der reversen DNA Polymerasen auf die Beeinflussung der DNA instruierten DNA Synthese zu untersuchen.

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der sich mehrenden Berichte über eine Störung der Zelldifferenzierung bei diesem Krankheitsbild als nicht therapeutisch zum Ziele führend erscheinen. Die Isolierung RNA-abhängiger DNA-Polymerasen aus Blutzellen [5, 6, 10] und onkogenen RNA-Viren wurde daher als *In-vitro*-Ansatz zur Auffindung antileukämischer Substanzen herangezogen. In der Zwischenzeit wurden verschiedenste Verbindungen ermittelt, welche die DNA-Polymerase der RNA-Viren hemmen [6, 10, 11]. In vielen Fällen wurde jedoch dabei ausser Betracht gelassen, dass diese Hemmstoffe möglichst spezifisch auf das virale Enzym wirken sollten, die normalen und zellständigen eukaryoten DNA-Polymerasen jedoch möglichst unbeeinflusst bleiben sollten. Die von uns untersuchten Rifamycinderivate scheinen dafür gewisse Voraussetzungen zu erfüllen und berechtigen zu weiteren Untersuchungen. Das Distamycinderivat mit der beobachteten Hemmwirkung auf DNA-abhängige DNA-Polymerasen erscheint dagegen als ein weit weniger spezifischer Hemmstoff. In diesem Zusammenhang ist es von Interesse, dass von verschiedenen Laboratorien über eine selektive Toxizität von Hemmstoffen der reversen Transkriptase auf transformierte Zellen berichtet wurde [8, 11].

Das Studium der DNA-Polymerasen normaler und leukämischer Zellen beansprucht daher in besonderer Weise experimentelles Interesse, um die pathophysiologischen Grundzüge der Leukämie besser verstehen zu können. Das Konzept, dass die in Leukämiezellen vorkommende reverse Transkriptase eine Rolle für das Initialstadium der Leukämie spielt und unter Umständen auch daran beteiligt ist, das Stadium der Malignität aufrechtzuerhalten, liesse daran denken, dass die Anwendung von Hemmstoffen der RNA-abhängigen DNA-Polymerasen ein neues therapeutisches Prinzip für die Behandlung maligner Erkrankungen darstellen könnte.

Diese Untersuchungen wurden mit Unterstützung des Österreichischen Forschungsrates (Projekt 1477) und im Auftrag des Österreichischen Bundesministeriums für Wissenschaft und Forschung durchgeführt.

Zusammenfassung

In leukämischen Leukozyten konnten 2 verschiedene DNA Polymerase Aktivitäten durch säulenchromatographische Auftrennung nachgewiesen werden, DNA abhängige DNA Polymerasen (Polymerase I und Polymerase II) und RNA abhängige Polymeraseaktivitäten. Die Template-(Primer)-Charakteristika dieser RNA

abhängigen DNA Polymerasen waren ähnlich wie für die reverse Transkriptase onkogener RNA Viren. Gewisse Unterschiede im Molekulargewicht RNA abhängiger DNA Polymerasen bei lymphatischer Leukämie gegenüber myelischer Leukämie sprechen für eine gewisse Heterogenität dieser Enzyme bei verschiedenen Leukämiespezies. Wesentlich ist der Unterschied zu normalen Blutzellen oder nicht leukämischen Blasten, da in diesen Zellen bisher niemals eine reverse Transkriptase nachgewiesen werden konnte. Die mögliche Beteiligung dieses Enzyms bei den Vorgängen der Malignität hat uns daher veranlaßt, in einem *In-vitro*-screening System verschiedene Hemmstoffe der reversen DNA Polymerasen auf die Beeinflussung der DNA instruierten DNA Synthese zu untersuchen.

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Le syndrome de Richter

Rapport de quatre observations et essai de dénombrement

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Abstract On the basis of 4 personal observations and a review of the literature the authors undertake an analysis of Richter's syndrome. Sometimes chronic lymphoid leukemia (CLL) and malignant reticulopathy (MR) are discovered simultaneously, sometimes MR is diagnosed after one or more years of development of a known CLL. In the latter case the appearance of the MR may be accompanied by persisting signs of the CLL, by their regression or disappearance. These differing situations probably have different meanings.

Key Words
Hodgkin's disease
Lymphatic leukaemia
Reticulosarcoma
Richter's syndrome

Depuis son individualisation par LORTHOIARY *et al* [1], le syndrome de Richter, associant une leucémie lymphoïde chronique (LLC) et une reticulopathie maligne (reticulosarcome ou maladie de Hodgkin), a fait l'objet de quelques publications rapportant de nouvelles observations en envisageant quelques hypothèses pathogéniques.

Ayant récemment observé quatre sujets atteints de cette maladie, il nous est apparu que l'appellation recouvrait en réalité des entités diverses, méritant d'être individualisées et répondant probablement à des pathogénies différentes. Ce que nous discuterons brièvement après avoir rapporté nos quatre observations personnelles qui illustrent cette diversité.

OBSERVATIONS

Observation I. Mlle B.
des adénopathies cervicales
typiques. Un traitement par
remission stable et de bonne

réponse. Découverte à l'âge de 43 ans, en 1964,
conduisant à la réalisation d'un diagnostic de LLC
appliqué pendant six mois, entraînant une
amélioration de l'état général de la malade.

présente une altération brutale avec fièvre III compression médiastinale d'origine ganglionnaire avec une polyadénopathie superficielle discrète. On constate alors une disparition de tout signe de LIC dans le sang comme dans la moelle et on évoque un syndrome de Richter qui est confirmé par une biopsie ganglionnaire cervicale affirmant un lymphoréticulocarcinome. Il s'agit d'un stade III_R qui fait l'objet d'une polychimiothérapie réductrice puis d'une irradiation en deux temps de toutes les aires ganglionnaires tronculaires, suivie d'une chimiothérapie d'entretien par vincristine. La malade reste en rémission apparemment complète quand elle décède en mars 1972 de décompensation d'une cardiopathie remontant à la petite enfance.

Observation 2 M. Rez (IB 71 1453) 67 ans est porteur depuis quatre ans d'une LIC stabilisée par administration continue de chlorambucil quand il est hospitalisé pour un tableau de décompensation avec fièvre et augmentation des adénopathies dans tous les territoires superficiels tandis que la lymphocytose reste stable autour de 20 000 mm³ avec 90% de petits lymphocytes au myélogramme. La lymphographie ayant révélé de très volumineuses adénopathies rétropectoraales on réalise d'abord une irradiation large de tous les territoires ganglionnaires sous diaphragmatiques. La fonte de ces adénopathies est rapide mais on observe simultanément une progression des ganglions cervicaux et axillaires. La biopsie d'un ganglion cervical permet d'affirmer l'existence d'un réticulocarcinome dictio-syncytial. Le malade reçoit alors une chimiothérapie associant vincristine, cyclophosphamide et prednisone. La régression tumorale atteint 70% mais est de brève durée et la reprise évolutive entraîne le décès du malade deux mois après l'apparition des signes de décompensation.

Observation 3 M. Fil (IB 71 1947) né en 1899 présente en avril 1971 un zona conduisant à la découverte d'adénopathies axillaires et cervicales modérées avec une lymphocytose sanguine à 15 000 mm³ et 80% de petits lymphocytes au myélogramme. Cette LIC paraît stable et n'est pas traitée. Un amaigrissement très important entraîne l'hospitalisation du malade début octobre 1971. Il présente une polyadénopathie diffuse et modérée avec une lymphocytose sanguine à 26 400. La biopsie d'un ganglion cervical permet d'affirmer un réticulocarcinome dictio-syncytial. Une dysphagie conduit à découvrir une sténose œsophagienne due à un carcinome épidermoïde peu différencié qui est irradié par cyclocoalthérapie. Simultanément est administrée une association de vincristine, cyclophosphamide et prednisone entraînant une régression ganglionnaire de 75%. A l'issue de son irradiation le malade rentre chez lui avec une chimiothérapie d'entretien mais la dysphagie qui a été peu améliorée s'accompagne d'une cachexie progressive responsable du décès du patient début janvier 1972 sans que se soit manifestée de reprise évolutive de son hémopathie.

Observation 4 M. Alp (IB 88 1286) 72 ans présente en juillet 1968 une altération générale qui conduit à découvrir une polyadénopathie prédominant en régions inguinales une lymphocytose sanguine à 54 600 et une infiltration massive de la moelle sternale par de petits lymphocytes. L'attention thérapeutique initiale est interrompue en janvier 1970 en raison d'une ascension de la lymphocytose au dessus de 100 000 mm³. Un traitement de six mois par chlorambucil rétablit une situation plus satisfaisante qui se maintiendra jusqu'en décembre 1971. A la fin de ce mois le malade est réhospitalisé avec une fièvre élevée une atteinte marquée de l'état général une croissance rapide de toutes les adénopathies une hépatoméno-

mégale importante et une lymphocytose sanguine à 94 000. La biopsie d'un ganglion cervical permet de porter le diagnostic d'une forme réticulaire de la maladie de Hodgkin. La chimiothérapie associant vinblastine, procarbazine et cyclophosphamide entraîne une chute de la lymphocytose à 15 000 mais la fonte tumorale est moins nette et le maintien d'un traitement d'entretien par vinblastine n'empêche pas une reprise évolutive en avril 1972, rapidement fatale.

Commentaires

Ces quatre observations étayées par une revue de la littérature, détaillée dans la thèse de VALRABOURO [2] amènent à distinguer, dans le cadre du syndrome de Richter, plusieurs types d'associations.

Dans un premier groupe d'observations les deux composantes du syndrome sont reconnues simultanément ou à quelques mois l'une de l'autre comme dans notre observation 3. Dans ce cas, il est impossible d'établir la chronologie exacte des deux perturbations. On ne peut exclure qu'il s'agisse d'une réaction leucémoïde lymphocytaire à une réticulopathie maligne, encore que ce type de réaction leucémoïde soit rare.

Pour les autres observations il existe un décalage important entre la reconnaissance initiale de la LLC et celle de la réticulopathie. (Les observations inverses de LLC survenant chez un malade suivi pour réticulopathie paraissent devoir être exclues du cadre du syndrome de Richter.) Dans ce cas plusieurs éventualités peuvent être schématiquement distinguées. Pour la plupart des cas, comme pour nos observations 2 et 4, la LLC persiste, inchangée, et l'on observe l'addition de la réticulopathie maligne. Il paraît logique alors d'assimiler cette situation à celle, plus générale et bien connue, de « LLC + cancer ». Cette tendance est appuyée par les quelques observations où un autre cancer s'ajoute au syndrome de Richter comme dans notre observation 3. Dans quelques observations au contraire on constate comme dans notre observation 1 une disparition spontanée des stigmates de la LLC au moment où se manifeste la réticulopathie donnant l'impression d'une substitution de la seconde maladie à la première et permettant seule d'évoquer une transformation de LLC en réticulopathie maligne d'évolution plus aigüe. Ces cas sont exceptionnels puisque nous n'en avons retrouvé que cinq autres dans la littérature. À côté de ces deux types bien tranchés se rangent toutes les observations où l'apparition de la réticulopathie maligne s'accompagne d'une réduction d'un signe de LLC et en particulier de la lymphocytose sanguine qui reste toutefois anormalement élevée. On ne peut actuelle-

présente une altération brutale avec fièvre et compression médiastinale d'origine ganglionnaire avec une polyadénopathie superficielle discrète. On constate alors une disparition de tout signe de LLC dans le sang comme dans la moelle et on évoque un syndrome de Richter qui est confirmé par une biopsie ganglionnaire cervicale affirmant un lymphoréticulosarcome. Il s'agit d'un stade III_B qui fait l'objet d'une polychimiothérapie réductrice puis d'une irradiation en deux temps, de toutes les aires ganglionnaires tronculaires, suivie d'une chimiothérapie d'entretien par vincristine. La malade reste en rémission apparemment complète quand elle décède en mars 1972 de décompensation d'une cardiopathie remontant à la petite enfance.

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Observation 3 M J (FB 71 1947) né en 1899 présente en avril 1971 un zona conduisant à la découverte d'adénopathies axillaires et cervicales modérées avec une lymphocytose sanguine à 15 900/mm³ et 80% de petits lymphocytes au myélogramme. Cette LLC paraît stable et n'est pas traitée. Un amaigrissement très important entraîne l'hospitalisation du malade début octobre 1971. Il présente une polyadénopathie diffuse et modérée avec une lymphocytose sanguine à 26 400. La biopsie d'un ganglion cervical permet d'affirmer un réticulosarcome dictio-synctial. Une dysphagie conduit à découvrir une sténose œsophagienne due à un carcinome épidermoïde peu différencié qui est irradié par cyclocoaltérapie. Simultanément est administrée une association de vincristine, cyclophosphamide et prednisone entraînant une régression ganglionnaire de 75%. A l'issue de son irradiation le malade rentre chez lui avec une chimiothérapie d'entretien mais la dysphagie qui a été peu améliorée s'accompagne d'une cachexie progressive responsable du décès du patient début janvier 1972 sans que se soit manifestée de reprise évolutive de son hémopathie.

Observation 4 M Alp (FB 68 1246) 72 ans présente en juillet 1969 une altération générale qui conduit à découvrir une polyadénopathie prédominant en régions inguinales, une lymphocytose sanguine à 54 600 et une infiltration massive de la moelle sternale par de petits lymphocytes. L'abstention thérapeutique initiale est interrompue en janvier 1970 en raison d'une ascension de la lymphocytose au dessus de 100 000/mm³. Un traitement de six mois par chlorambucil rétablit une situation plus satisfaisante qui se maintiendra jusqu'en décembre 1971. A la fin de ce mois le malade est rehospitalisé avec une fièvre élevée, une atteinte marquée de l'état général, une croissance rapide de toutes les adénopathies, une hépatomégalo-

Tabelle 1 Halbquantitative Enzymaktivitätsbestimmung in Megakaryozyten der Ratte bei unterschiedlicher Dosierung von Cyclophosphamid

| Dosis
mg/kg KG | Reaktionsstufen | | | | | | | | | | | | | | |
|-------------------|---------------------|----|----|----|---|------------------------|----|----|---|---|-------------------|----|----|---|---|
| | Laktatdehydrogenase | | | | | Alkalische Phosphatase | | | | | Saure Phosphatase | | | | |
| | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 |
| 1 × 10 | 0 | 1 | 11 | 8 | 5 | 0 | 8 | 9 | 5 | 3 | 9 | 7 | 7 | 2 | 0 |
| 1 × 25 | 0 | 5 | 17 | 3 | 0 | 5 | 13 | 5 | 0 | 0 | 4 | 12 | 9 | 0 | 0 |
| 1 × 50 | 0 | 3 | 15 | 6 | 1 | 13 | 10 | 2 | 0 | 0 | 1 | 5 | 18 | 4 | 1 |
| 1 × 75 | 1 | 6 | 10 | 6 | 2 | 8 | 11 | 6 | 0 | 0 | 1 | 6 | 13 | 5 | 0 |
| 1 × 100 | 0 | 10 | 11 | 4 | 0 | 9 | 10 | 6 | 0 | 0 | 0 | 5 | 16 | 4 | 0 |
| Kontrollen | 0 | 0 | 11 | 8 | 6 | 6 | 8 | 10 | 1 | 0 | 16 | 6 | 3 | 0 | 0 |
| 2 × 10 | 2 | 9 | 8 | 3 | 1 | 12 | 5 | 7 | 1 | 0 | 3 | 5 | 9 | 8 | 0 |
| 2 × 25 | 0 | 2 | 12 | 11 | 0 | 10 | 11 | 4 | 0 | 0 | 9 | 11 | 5 | 0 | 0 |
| 2 × 50 | - | - | - | - | - | 9 | 9 | 6 | 1 | 0 | 3 | 12 | 11 | 0 | 0 |
| 2 × 75 | - | - | - | - | - | 13 | 9 | 3 | 0 | 0 | 10 | 9 | 4 | 2 | 0 |
| 2 × 100 | - | - | - | - | - | 13 | 9 | 3 | 0 | 0 | - | - | - | - | - |

LDH, AP und SP waren in Megakaryozyten der Ratte zytochemisch nachweisbar. Aktivitätsveränderungen der drei Enzyme setzten synchron mit dem Thrombozytenabfall im peripheren Blut schon bei niedrigster Dosierung von Cyclophosphamid (10 mg/kg Körpergewicht) ein. Nur bei der LDH konnten wir mit steigender Dosierung eine Korrelation von Aktivitätsverlust und Thrombozytenzahl finden.

1 LDH Nach einmaliger Injektion von 10, 25, 50, 75 und 100 mg Cyclophosphamid/kg Körpergewicht (KG) sank die LDH Aktivität linear leicht ab (Abb. 1, Tab. 1). Bei Dosierungen von 200, 250 und 500 mg/kg KG erfolgte der Abfall steil. Bei 500 mg/kg KG zeigte sich in den wenigen noch vorhandenen Megakaryozyten keine Aktivität mehr. Der Aktivitätsverlust war in Zellkern und Zytoplasma gleichmäßig (Abb. 2). Bei zweimaliger Injektion von 10 und 25 mg/kg KG wurden noch mittlere Aktivitäten (bis Reaktionsstufe 3) beobachtet. Dagegen war nach zweimaliger Injektion von mehr als 50 mg/kg KG Aktivität nur noch in Spuren vorhanden. Die Abnahme des Enzyrgehaltes unter Cyclophosphamid ist nach unserer Auffassung auf eine Syntheschemmung zurückzuführen, die sich ihrerseits durch RNA-Hemmung erklären lässt. Untersuchungen am Rattenboden [5] führten zu vergleichbaren Ergebnissen.

2 AP und SP Beide Enzyme verhielten sich in ihrer Dosis-Aktivitäts-Relation unterschiedlich. Bei der AP wurde unter geringen Cyclo-

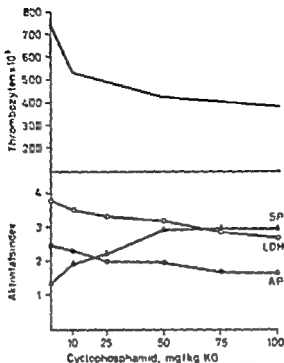


Abb 1. Verhalten der Enzyme LDH, AP und SP in Megakaryozyten sowie der Thrombozyten im peripheren Blut unter verschiedenen Cyclophosphamiddosen

tronics, Krefeld) gezählt 26 Ratten wurde einmal, weiteren 26 zweimal im Abstand von 24 h Cyclophosphamid i.p. in unterschiedlicher Dosierung appliziert 24 h nach der letzten Injection wurde nach nochmaliger Thrombozytenzählung Knochenmark aus dem Femur punktiert Die Punktate wurden ausgestrichen, luftgetrocknet, in Aceton fixiert und histochemisch auf LDH nach FRANK [7], AP nach WACHSTEIN [12] und SP nach GEMORI [3] untersucht 20 Tiere des gleichen Stammes blieben unbehandelt und dienten als Kontrollen

In Anlehnung an VITZKE und ZURWITTE [6] wurden die Ausstrichpräparate halbquantitativ ausgewertet Jeweils 25 Megakaryozyten wurden ausgezählt und nach Intensität der histochemischen Reaktion 5 Aktivitätsstufen zugeordnet

Ergebnisse und Diskussion

Wie bereits von anderen Autoren beschrieben [1, 4], fanden wir nach Cyclophosphamid eine deutliche Depression aller Arten von Knochenmarkszellen Die Megakaryozytenzahl war um so niedriger, je höher die Cyclophosphamiddosis war Dieses Verhalten lässt sich durch Hemmung der DNS-Synthese erklären

Some Aspects of Leucocyte Behaviour in Haemodialysis

LUGI BUSCARINI and FRANCESCO BASSI

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Abstract Neutropenia early in haemodialysis is probably due to the return into the circulation of leucocytes damaged by the first contact with the dialyzing surface. The phenomenon finishes because the damaging action of the membranes may be rapidly eliminated by a protective film of haematic derivations. We did not observe any modifications of the leucocytes on re using (in the same dialysis) the same dialyzer, provided it is not rinsed. The leucocyte values immediately before the end of dialysis are lower than the starting values. This phenomenon is due to the progressive sedimentation of white cells on the dialyzing membranes during haemodialysis.

Key Words
Haemodialysis
Leucocytes in
haemodialysis
Leucopenia

In 1965, KAMOW and GOTTINET [3] described a profound transient neutropenia with fall of monocytes early in haemodialysis, the number of lymphocytes remaining the same. The phenomenon was ascribed to a transient sequestration *in vivo*, principally in the lungs. The leucopenic phase is followed by rapid recovery with rebound 1-3 h after the beginning of dialysis. The recovery is due to a re-appearance of sequestered granulocytes and to a significant response of the bone marrow. The phenomenon is constant, independent on the type of kidney disease, on the number of dialysis performed, on the type of dialyzer used. It also appears in a healthy dialyzed dog. It is caused neither by heparin, nor by initial bleeding, nor by extracorporeal circulation (in fact, it does not appear by-passing the dialyzer). The phenomenon is induced neither by rapid infusion of 150-200 ml of dialyzing fluid which issues from the compartment of the dialyzer, nor by infusion of blood that has previously been in contact with the dialyzer. Neutropenia can be reproduced during the same dialysis by changing the dialyzer after the white count has returned to normal [1, 3-6].

The first part of the paper is devoted to a discussion of the
 various methods which have been proposed for the determination of
 the rate of reaction between a radical and a molecule. The
 most common method is the use of a stopped-flow apparatus,
 in which the reaction mixture is rapidly mixed and the
 reaction is allowed to proceed for a short time before the
 reaction is stopped. The concentration of the reactants is
 then determined by a suitable analytical method. The rate
 of reaction is then calculated from the change in concentration
 of the reactants over the time interval. Another method is
 the use of a laser flash photolysis apparatus, in which a
 laser pulse is used to generate a radical, and the reaction
 is allowed to proceed for a short time before the reaction
 is stopped. The concentration of the reactants is then
 determined by a suitable analytical method. The rate of
 reaction is then calculated from the change in concentration
 of the reactants over the time interval. A third method is
 the use of a continuous-flow apparatus, in which the
 reaction mixture is continuously mixed and the reaction is
 allowed to proceed for a short time before the reaction is
 stopped. The concentration of the reactants is then
 determined by a suitable analytical method. The rate of
 reaction is then calculated from the change in concentration
 of the reactants over the time interval.

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Key Words
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haemodialysis
Leucopenia

In 1968 KAMLOW and GORTNER [3] described a profound transient neutropenia with fall of monocytes early in haemodialysis, the number of lymphocytes remaining the same. The phenomenon was ascribed to a transient sequestration *in vivo* principally in the lungs. The leucopenic phase is followed by rapid recovery with rebound 1-3 h after the beginning of dialysis. The recovery is due to a re appearance of sequestered granulocytes and to a significant response of the bone marrow. The phenomenon is constant, independent on the type of kidney disease, on the number of dialysis performed, on the type of dialyzer used. It also appears in a healthy dialyzed dog. It is caused neither by heparin, nor by initial bleeding nor by extracorporeal circulation (in fact, it does not appear by-passing the dialyzer). The phenomenon is induced neither by rapid infusion of 140-200 ml of dialyzing fluid which issues from the compartment of the dialyzer nor by infusion of blood that has previously been in contact with the dialyzer. Neutropenia can be reproduced during the same dialysis by changing the dialyzer after the white count has returned to normal [1, 3, 4].

The purpose of the present paper is to try to clarify the dynamics of the phenomenon. Moreover, following our observation [2] of a progressive leucocyte sedimentation on the dialyzing membranes during haemodialysis, we have studied the behaviour of leucocytes at the end of dialysis.

Material and Methods

In 16 patients dialyzed twice a week (table I) the white cell count was done on microscopy before the start of dialysis, 20 min after, immediately before the end and 15 min after the end.

In 5 of these patients the count was also performed before and after the change of dialyzer (at about the second hour of dialysis). On these same patients we also carried out the following experiments: interruption of dialysis at the second hour, recovery of blood by air, immediate restarting of dialysis with the same dialyzer which we re-used without washing on one occasion and on another occasion after washing with 1 000 ml of 0.9% sodium chloride. The white count was performed before the interruption and during the phase after the reconnection of the patient.

Finally we have studied the behaviour of leucocytes also in a healthy man submitted to a dialysis lasting 25 min (circulation vein to vein with pump, blood flux in dialyzer 60-70 ml/min).

Results

In the first phase of haemodialysis, a remarkable fall of granulocytes and monocytes appears in our uraemic patients, on the contrary, there is a low fall of lymphocytes. The same kind of changes in the white cell count are seen in a healthy dialyzed man. In patient No. 13 (treated with vinblastine), the fall in the white count is much lower than in all other patients, it is practically non-existent in a second study performed when the starting neutrophil values are much more reduced. The leucocyte values immediately before the end of dialysis are lower than the starting values, 15 min after blood recovery, we can see a constant and significant increase of leucocytes which reach the pre-dialysis levels (table I).

We confirm that the phenomenon re-appears more than once during the same dialysis by changing the dialyzer after the white count has returned to normal. Neutropenia, on the contrary, does not appear again if the patient is reconnected to the same dialyzer after blood recovery. The phenomenon manifests itself in such a situation if the dialyzer is rinsed before re-using (fig. 1).

Table 1 Haemodialysis

| Case no. | Age years | Sex | Months of dialysis | Diagnosis | Leucocytes, mm ³ (granulocytes, mm ³) | | | |
|----------|--------------|-----|--------------------|-----------------------------|--|---------------|---------------|---------------|
| | | | | | I | II | III | IV |
| 1 | 41 | M | 33 | chronic pyelonephritis | 5 500 (4 000) | 1 100 (180) | 5 000 (3 500) | 6 800 (4 600) |
| 2 | 42 | M | 23 | chronic nephritis | 3 400 (2 700) | 1 000 (200) | 2 000 (1 500) | 3 100 (2 600) |
| 3 | 43 | M | 3 | chronic nephritis | 6 100 (4 600) | 1 200 (300) | 4 900 (3 400) | 5 700 (4 200) |
| 4 | 43 | M | 8 | chronic nephritis | 5 600 (3 300) | 1 100 (350) | 5 000 (3 000) | 6 800 (4 200) |
| 5 | 42 | M | 11 | chronic nephritis | 5 200 (4 100) | 900 (200) | 3 700 (2 800) | 5 000 (4 000) |
| 6 | 45 | M | 12 | chronic nephritis | 4 400 (3 100) | 3 000 (260) | 3 000 (2 000) | 4 100 (3 200) |
| 7 | 32 | M | 10 | chronic nephritis | 5 000 (3 400) | 1 100 (300) | 2 800 (1 700) | 5 000 (3 300) |
| 8 | 40 | F | 16 | pyelonephritis & kidney | 5 800 (4 900) | 700 (160) | 4 300 (3 200) | 5 800 (4 600) |
| 9 | 19 | F | 44 | chronic nephritis | 6 000 (4 800) | 1 000 (100) | 4 300 (3 200) | 4 300 (3 300) |
| 10 | 11 | M | 70 | chronic nephritis | 7 000 (5 700) | 1 200 (210) | 4 000 (3 400) | 6 500 (5 200) |
| 11 | 50 | M | 16 | chronic nephritis | 4 100 (3 200) | 2 800 (1 200) | 3 800 (3 000) | 4 400 (3 300) |
| 12 | 44 | M | 5 | chronic nephritis | 5 100 (3 900) | 1 400 (300) | 4 000 (3 300) | 4 900 (3 800) |
| 13 | 24 | M | 18 | cortical necrosis & Hodgkin | 2 500 (1 500) | 1 000 (700) | 1 600 (1 000) | 3 700 (2 400) |
| 14 | second study | | | | 1 200 (1 000) | 900 (700) | - | - |
| 15 | 42 | M | 33 | amyloid & kidney | 7 000 (5 000) | 1 000 (100) | 3 000 (1 950) | 5 800 (3 900) |
| 16 | 57 | F | 0.5 | chronic nephritis | 8 100 (6 600) | 1 700 (400) | 6 100 (4 800) | 8 000 (6 500) |
| 17 | 39 | M | - | healthy | 8 400 (5 900) | 3 300 (1 200) | - | - |

Two weeks, patients undergo haemodialysis lasting from 11 to 12 h with KI 1 kidney. White count is done on morning before the start of dialysis (I), 70 min after (II), immediately before the end (III), and 15 min after the end (IV). Before and after the end we have not observed any heart road sections in the 2 percent of white count. In the healthy man we see the usual fall in neutrophils early in haemodialysis. Granulocyte values in parentheses.

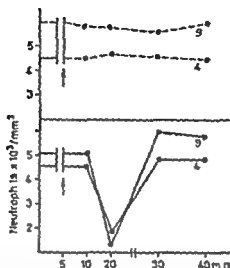


Fig 1 The re use of the same dialyzer late in haemodialysis (recovery of blood by air without washing) does not induce any leucocyte modifications (upper panel) The neutrophils fall if the dialyzer is washed before re use (lower panel) Experiments were performed in the second hour in 2 different haemodialyses The arrows indicate the time of reconnection of the patient to the dialyzer (9 and 4 = case No., see table I)

Discussion

Neutropenia early in haemodialysis, due to a reversible sequestration *in vivo*, appears after the flux of a critical amount of blood through the dialyzer. The phenomenon ends spontaneously. It re-appears during the same dialysis when the patient is connected to a fresh dialyzer. The re-use of the same dialyzer (late in dialysis) does not cause neutropenia, it appears, on the contrary, if the dialyzer is washed before re-use.

We can formulate a work hypothesis which explains the different aspects of the phenomenon – defined 'a fascinating puzzle' by SMITH and JOHNS [5]. The sequestration *in vivo* (which induces neutropenia) could be a self multiplying phenomenon caused by the return to the patient of an amount of leucocytes which have been modified by the first contact with the membranes – we recall the experimental leucopenia by transfusion of altered leucocytes [7]. The process finishes because the damaging power of the dialyzing surface comes rapidly to an end. The demonstration that the re-use of the same dialyzer does not cause leucocyte modifications, provided the chambers are not rinsed, suggests that a pro-

fective film from the blood eliminates the damaging properties of the membranes: a simple washing can alter this layer.

The second aspect of leucocyte behaviour in haemodialysis focused by us is the final decrease: our data agrees with those published by PAPADIMITRIOU *et al* [4].

We think that this phenomenon depends on the sequestration of leucocytes in the dialyzer during haemodialysis [2].

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Congenital Hypoproconvertinemia (Factor VII Deficiency)

A Report of Two Cases Belonging to Two Different Kindreds

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Abstract Two new cases of congenital factor VII deficiency are reported. They are two 7-year-old boys belonging to two different kindreds. The main clinical features were bleeding from the gums, epistaxis (one patient), easy bruising, melena, post-traumatic and spontaneous hemarthrosis. The main coagulation features were prolonged prothrombin time, normal Stypven-cephalin clotting time, normal first stage coagulation tests, normal platelet and vascular tests. The prothrombin time was corrected by the addition of normal serum factor II or factor V or factor X-deficient plasmas and of the abnormal factor X (factor X Friuli) plasma. On the contrary, the addition of adsorbed normal plasma and of three plasmas with known factor VII deficiency failed to correct the abnormality. The factor VII levels were found to be 2.3 and 2.7% of normal, respectively. The parents of the patients were not consanguineous. But 3 of them, together with other members of the 2 families, were found to have slightly decreased factor VII levels and were considered to be heterozygotes for the defect. These findings are consistent with an incompletely recessive pattern of autosomal inheritance.

Key Words
Bleeding disorders
Blood coagulation
Factor VII deficiency
Hypoproconvertinemia

Congenital factor VII deficiency is still considered to be a rare coagulation disorder. So far, only about 56 sufficiently proved cases have been reported in the world literature. 39 'proved' or 'probable' cases were gathered by OWEN *et al* in 1964 [21]. We found 11 additional cases in a reperusal of the papers appearing before or during 1964 [2, 7, 8, 13, 19]. Since 1965, only 6 cases have been reported [6, 12, 18, 20]. Several 'probable' cases of factor VII defect have been described, but final proof of the nature of the defect in these cases is lacking [21]. This

is particularly true for the cases reported before 1956 or 1957, the years in which factor X defect was first recognized. The matter is further complicated by the recent finding that one of the criteria commonly maintained to indicate a factor VII defect (a normal Russell's viper venom clotting time together with a prolonged prothrombin time) is no longer true [4]. In fact, in the abnormal factor X (factor X Friuli) coagulation disorder, the Stypven-cephalin clotting time is normal whereas the prothrombin time is prolonged and the factor VII level is normal. The peculiar behavior is due to the fact that the abnormal factor X can be activated normally by Russell's viper venom [4].

The object of this paper is to report on 2 patients with pure factor VII congenital defects together with family studies. These are the fifth and the sixth patients with this defect reported in Italy. The previous patients were reported recently by others and by us [6, 20].

Materials and Methods

Material and method used were reported in detail elsewhere [3, 4]. Only new data will be given herein.

Three factor VII-deficient plasmas were used in cross experiments and in factor VII assay. The first plasma was kindly supplied in lyophilized form by Dr. C. Owen, Department of Pathology, Mayo Clinic, Rochester, Minn., USA. Another lyophilized factor VII-deficient plasma was supplied by Dade Laboratories, Miami, Fla., USA. We also used the noncontacted frozen plasma of another patient with congenital hypoproconvertinemia [6].

All factor VII assays were carried out using as substrate equal parts of lyophilized Dade factor VII-deficient plasma and adsorbed normal plasma. All the test plasmas assayed were noncontacted plasmas but the assay was carried out in normal glassware.

Case 1, a 7 year-old boy, was first seen by us in 1962.

The family history was negative for bleeding disorders, and the parents were not consanguineous (Fig. 1). The patient was born in term, and no undue bleeding was noted in the neonatal period. With the appearance of the first teeth, ecchymoses of the gums was noted.

At the age of 1½ years, the patient underwent minor surgery for the correction of a phimosis. Only moderate bleeding was noted.

At 2 years, the patient started complaining of frequent epistaxis and had to be admitted several times to a local hospital.

At 3 years, the patient presented a large post-traumatic hematoma of the right flank and had to be admitted to another hospital. Several whole blood transfusions were given, and the hematoma slowly subsided.

- = proband homozygote, symptomatic;
 □ or ○ = heterozygotes asymptomatic;
 □ or ○ = studied asymptomatic, normal;
 □ or ○ = not studied asymptomatic, normal?

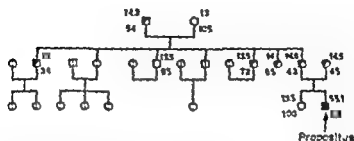


Fig 1 Family pedigree of case 1. The upper numbers on the right or left side of each square (male) or circle (female) refer to the prothrombin time in sec, the lower numbers refer to the factor VII percentile level.

- = proband homozygote symptomatic;
 □ or ○ = heterozygotes asymptomatic;
 □ or ○ = studied, asymptomatic normal;
 □ or ○ = not studied asymptomatic normal?
 ■ = deceased, asymptomatic, normal?

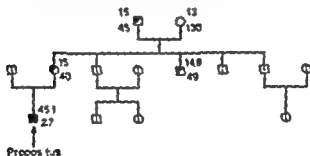


Fig 2 Family pedigree of case 2. The numbers have the same meaning as in figure 1.

During the past 3 years the patient has suffered several bleeding episodes: melena, hematuria, posttraumatic hemarthrosis of both elbows, and a posttraumatic hemarthrosis of the left knee. On every occasion the patient had to be hospitalized and was treated with whole blood or plasma transfusions. No functional limitations of the joints remained after the hemarthrosis. During the last admission a tentative diagnosis of factor VII deficiency was formulated, and the patient was sent to us for further studies.

Case 2 a 7 year-old boy, was also first seen by us in 1972.

The patient was born at term to an unwell mother, and no bleeding disorder was noted at birth. Family history on the maternal side was negative for bleeding disorder. The father was not available for information but no bleeding tendency seemed to be present in his family either (Fig. 2).

The patient started complaining of bleeding from the gums at the age of 2½ years. At 3 years the patient started to bruise easily. Epistaxis on the other hand, was rare. At 5 years, the patient bled severely from the gums, experienced mild hematuria, and had to be admitted to a local hospital. The patient was transfused with whole blood or plasma, and bleeding subsided promptly.

Since the age of 5 years, the patient has presented several episodes of hemarthrosis in practically all major joints, but particularly in the left elbow. These hemarthroses usually followed traumas of variable intensity; occasionally no trauma history could be elicited and the episodes were considered spontaneous. The repeated hemarthrosis of the left elbow led to a mild to moderate ankylosis of the joint.

At 6 years the patient presented another episode of gingival bleeding which required another hospital admission. During the past year he presented hemarthroses of both ankles and 2 episodes of posttraumatic bleeding from the gums. In all instances the patient had to be hospitalized and was treated with whole blood or plasma transfusions.

No bleeding manifestations were prevented at the time of study. Physical examination revealed the presence of a slight underweight and a limited function of the left elbow joint. The patient was able to extend the arm only about 160°.

Results

The results of the coagulation studies are summarized in tables I-III. The prothrombin time was prolonged and corrected by addition of normal plasma, though not by addition of adsorbed normal plasma. Plasma congenitally deficient in factor II or V or X and plasma with the abnormal factor X (factor X Fushi) disorder corrected the abnormality. Yet 3 plasmas with known factor VII deficiency failed to correct it. The prothrombin time of plasma mixture was also prolonged in the 2 cases. Glass clotting time, recalcification time and the thromboelastogram were perfectly normal. Partial thromboplastin time, prothrombin consumption and the thromboplastin screening test were normal too. The thromboplastin generation test was normal or showed a very mild serum defect (fig. 3). Platelet tests and bleeding time were within normal limits, and there was no hyperfibrinolysis.

The factor VII level found in the plasma of our cases was 2.3 and 2.7% of normal, respectively. Both parents of case 1 and the mother of

Table 1 Complete coagulation studies in case 1. Similar results were obtained in case 2

| | Case 1 | Normal values |
|-------------------------------------|-------------------------------|-----------------------|
| Platelet count | 250 000 | 150 000-350 000 |
| Bleeding time, min | 3 ¹ / ₂ | 2-5 |
| Clot retraction | complete after 6 h | complete after 12 h |
| Glass clotting time, min | 8 ¹ / ₂ | 5-10 |
| Partial thromboplastin time, sec | 40.5 | 35-45 |
| Prothrombin consumption, sec | >90 | >90 |
| Thromboplastin screening test | 16 sec in 8 min | <15 sec in 6 or 8 min |
| Thromboplastin generation test | 18 sec in 8 min | <16 sec in 6 or 8 min |
| PT, sec | 55.1 | 13-14 |
| PP test (Owen), sec | 91.6 | 26.6 |
| Stypven-cephalin clotting time, sec | 12 | 11-13 |
| Thrombin time, sec | 11 | 18-25 |
| Fibrinogen, mg% | 310 | 250-450 |
| Factor II (one stage) % | 95 | 85-115 |
| Factor II (two-stage) % | 95 | 85-115 |
| Factor V, % | 100 | 85-115 |
| Factor VII, % | 2.3 | 85-115 |
| Factor VIII, % | 110 | 70-150 |
| Factor IX, % | 100 | 80-130 |
| Factor X, % | 100 | 85-115 |
| Factor XI, % | 120 | 60-150 |
| Factor XII, % | 110 | 60-150 |
| Thromboelastogram | | |
| r, mm | 17 | 10-20 |
| K, mm | 6 | 6-12 |
| ma, mm | 62 | 40-66 |

case 2 together with other family members had slightly decreased factor VII values and were considered to be heterozygous for the abnormality. These heterozygotes were all asymptomatic (fig. 1, 2).

Discussion

The main criteria for diagnosis of factor VII deficiency are (1) prolonged prothrombin time corrected by addition of normal serum, (2) normal or near normal first-stage tests, and (3) normal Stypven-cephalin clotting time. Our patients meet these criteria fully. The lack of correc-

Table II Prothrombin time correction studies in case I. Similar results were obtained from the other patient

| Mixture,
equal parts | Clotting time
sec | Reference
plasma PT
sec | Comment |
|---|----------------------|-------------------------------|--|
| Plasma | 55.1 | | |
| Plasma + adsorbed
normal plasma | 46.0 | | |
| Plasma
+ normal serum | 17.3 | | |
| Plasma + factor VII-deficient
plasma | 54.0 | 45.0 | Lyophilized factor VII-
deficient plasma
received from Dr. Owren |
| Plasma + factor VII-deficient
plasma | 44.5 | 40.1 | Dade lyophilized factor
VII-deficient plasma |
| Plasma + plasma of another patient
with factor VII deficiency | 53.0 | 43.5 | Noncontacted frozen
plasma, personal case |
| Plasma + factor X-deficient
plasma | 16.5 | 72.5 | Dade lyophilized factor
X-deficient plasma |
| Plasma + abnormal factor X
coagulation disorder
(factor X Friuli) | 16.2 | 33.3 | Frozen plasma, personal
case |
| Plasma + factor II-deficient
plasma | 16.0 | 21.0 | Frozen plasma, personal
case |
| Plasma + factor V-deficient
plasma | 15.8 | 43.0 | Frozen plasma, personal
case |

tion of the prothrombin time after addition of the plasmas of 3 known cases of factor VII deficiency establishes the diagnosis unequivocally. The slight abnormality observed in the thromboplastin generation system found in one of our cases is not surprising since this has already been seen in other proved factor VII deficient cases [6, 21]. This could indicate that factor VII plays a minor role even in the thromboplastin generation. The studies which deal with the relationship between the

Table III Main laboratory findings in case 2. Unreported findings were within normal limits

| | Case 2 | Normal values | Comment |
|---|--------------------|--------------------------|---|
| PT, sec | 45.1 | 13 | |
| Factor VII % | 2.7 | 85-115 | |
| Partial thromboplastin time, sec | 39 | 35-45 | |
| Stypven-cephalin clotting time sec | 13 | 11-13 | |
| PT mixture patient plasma +
case 1 plasma, sec | 48 | | Case 1 plasma was
non-contacted
frozen plasma |
| Thromboplastin generation test | 16 sec
in 8 min | <16 sec in
6 or 8 min | |

blood-coagulation contact-phase system and factor VII seem to support this contention [16]

There are no typical bleeding manifestations in patients with factor VII deficiency. The severity of bleeding is variable. Epistaxis, bleeding from the gums, easy bruising and menorrhagias are the commonest bleeding manifestations [21]. Hemarthroses are common but are often posttraumatic. The frequency of hemarthrosis seems to be definitely higher than in other prothrombin complex factor deficiencies.

OWEN *et al* [21] have postulated that 2 types of factor VII deficiency exist on the basis of an early or late onset of bleeding symptoms. However, this assumption is incorrect, since it was based on the data concerning 2 patients who were thought to have factor VII deficiency. They were subsequently found to have the abnormal factor X (factor X Friuli) coagulation disorder [4].

The occurrence of bleeding in the patients with congenital factor VII deficiency is puzzling since the vascular, platelet and intrinsic coagulation systems are normal. The coagulation defect consists only in an abnormally slow activation of factor X by the tissue thromboplastin + factor VII complex. The most likely explanation is to admit that a good hemostasis *in vivo* is dependent on the presence of adequate proconvertin level. Whether this is due to the fact that tissue thromboplastin plays an important role *in vivo* or due to some other unidentified mechanism remains to be proven.

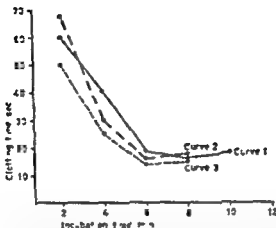


Fig 3 Thromboplastin generation test in case 2. The pattern is normal. Curve 1 is the basal curve. Curve 2 was obtained after the substitution of patient's adsorbed plasma with adsorbed normal plasma. Curve 3 was obtained after the substitution of patient's serum with normal serum. In case 1 a mild serum defect was noted.

Consanguinity was not present among the parents of our patients. This is not unusual since it was already seen in other cases [6, 21]. The pattern of inheritance seems to be incompletely recessive in autosomal type [6, 17, 23]. This assumption is fully confirmed by the present findings. The parents of case 1 and the mother of case 2 as well as other members of the family had decreased factor VII levels and were therefore considered heterozygotes. The father of case 2 is probably a heterozygote too. If he is not, a mutation has to be assumed to explain the heterozygosity found in his son. Heterozygotes may be easily differentiated from the normal population on the basis of a specific factor VII assay.

However, our heterozygotes also almost always showed a 1- to 2-sec prolongation of the prothrombin time. In this regard, it is interesting to note that heterozygotes for factor VII deficiency seem to be completely asymptomatic. We have seen 12 heterozygotes for this defect (9 related to the patient and 3 belonging to another family) [6]. None of them has noticed undue bleeding, even after surgical procedures, parturition, or trauma. This aspect is rather strange—and puzzling too, since heterozygotes have been reported to bleed unduly on occasion [1]. However, more

surgery or trauma [3-5, 10] related to other prothrombin complex factor deficiencies or abnormalities such as congenital hypoprothrombinemia, factor X deficiency, and the factor X Friuli coagulation disorder. Since the lowest factor VII levels found in these heterozygotes was about 34-45% of normal, it could be speculated that such levels are hemostatically sufficient.

Finally it has been shown recently that some factor VII-deficient plasmas contain anti-factor VII-reacting material [9]. These observations, if confirmed, would indicate the existence of genetic variants for the factor VII defect. Data concerning the genetic variants of the other prothrombin complex coagulation disorders have already been firmly established [4, 14, 16].

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surgery or trauma [3-5, 10] related to other prothrombin complex factor deficiencies or abnormalities such as congenital hypoprothrombinemia, factor X deficiency, and the factor X Friuli coagulation disorder. Since the lowest factor VII levels found in these heterozygotes was about 34-45% of normal, it could be speculated that such levels are he-
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Finally it has been shown recently that some factor VII-deficient plasmas contain anti-factor VII-reacting material [9]. These observations, if confirmed, would indicate the existence of genetic variants for the factor VII defect. Data concerning the genetic variants of the other prothrombin complex coagulation disorders have already been firmly established [4, 14, 16].

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Platelet Defect in a Case of Ehlers-Danlos Syndrome

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Abstract In a case of Ehlers Danlos syndrome, a platelet defect is described. It is characterized by a defect of ADP and platelet factor 3 release, absence of secondary curve in ADP, adrenalin and distilled water induced aggregation and a defective collagen aggregation. It is pointed out that this defect, which occurs in primary thrombopathy, also exists in connective tissue diseases such as Ehlers Danlos syndrome, osteogenesis imperfecta, etc.

Key Words
ADP of platelets
Connective tissue diseases
Ehlers Danlos syndrome
Platelet aggregation
Platelet factor 3
Thrombopathy

The Ehlers-Danlos syndrome is a rare hereditary connective tissue disease characterized by hypermobility of joints, hyperelasticity of skin, easy bruising and hemorrhagic tendency [5, 9, 18]. It is genetically determined and transmitted by the autosomal dominant mode of inheritance. Bruising tendency is frequently present and orthopedic, gastrointestinal, cardiovascular and ocular disturbances may occur. Other features which are sometimes found are calcified subcutaneous spheroids and molluscoid pseudotumors.

Although the presence of hemorrhagic tendency in this syndrome has long been observed, our knowledge is still limited regarding its pathogenesis [24]. LISXER *et al* [16] have reported factor IX deficiency in 2 cases of Ehlers Danlos syndrome. On the other hand, platelet defects have also been reported by GOODMAN *et al* [8], KASHIWAGI *et al* [13] and ESTES [7].

In this paper, a case of Ehlers Danlos syndrome associated with a platelet defect is presented.

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Methods

Platelets were counted according to the method of BARZAN and CROVITZ [3]. Clotting time was performed by the method of IYR *et al* [12]. Coagulation time was determined by the method of LEE and WHITE [15]. One-stage prothrombin time was measured by the method of QUICK [20] and two-stage prothrombin time was determined by the method of WARE and SEIGERS [32]. Fibrinogen determination was performed according to RATNOFF and MENZIE [21]. Partial thromboplastin time was determined by the method of LANDRELL *et al* [14]. Platelet factor 3 availability was performed by the method of SPART and COITROW [23]. Platelet factor 3 activity was determined using modified thromboplastin generation test [2, 30] (the platelet counts in the final solutions were adjusted to 100,000/mm³). Erythrocyte lysis time was performed as described by CORLEY *et al* [10]. Factor VIII assay was determined by the method of PITNEY [19] and factor IX with the method of BARROW and GRAM [1]. Factor XIII determination was done using the method of LORAND and DICERMAN [17] and SING's method [22].

Platelet aggregation studies were done using Bryton aggregometer (Bryton Manufacturing Ltd, Rexdale, Ont., Canada) and Baugh and Lamb recorder. For aggregation curves in the final solution, ADP was 0.5 µg/ml and adrenaline was 25 µg/ml. Distilled water induced aggregation was performed as described by ULTRY and ULTRY [31]. Collagen was obtained and prepared from the human rectus abdominus fascia.

ADP determination of platelet was done by using Boehringer enzyme sets and read at 366 nm (UV) with Beckman DU 2 spectrophotometer.

Case Report

The patient is a 47 year-old male. He was complaining of pain in both hips, elbows and dorsal region. He had been a full term baby and his birth had been normal. Neither his parents nor any of the known relatives had any signs of the Marfan's syndrome. He was complaining ever since his childhood of easy bruising and ecchymosis after slight trauma. He suffered from a hypermobility of joints in his childhood. He had limitation of motion secondary to frequent sprains and strains followed by blood effusions and swelling. A gastrointestinal bleeding occurred when he was 43 years old.

Mild kyphoscoliosis. Limitation of flexion and extension of lumbar spine. Loss of physiologic lumbar lordosis. There was an old rupture at the suprapubic junction of the right vastus medialis muscle. Several subcutaneous painless nodules of different size were present at the dorsal aspect of elbow bilaterally. There was also limitation of flexion and extension at both elbow joints.

Electromyography (with computer control) of radial and flexor nerve conduction was found to be within normal limits.

X-ray examination revealed calcified subcutaneous nodules in the region of great trochanter and olecranon bursa bilaterally. Heads of radius were dislocated and

Table I Laboratory findings

| | |
|--------------------------------|-------------------------|
| Bleeding time, min | ■ |
| Coagulation time, min | 5-6 |
| Retraction | normal |
| Platelet count | 200 000/mm ³ |
| Prothrombin time, sec | 15 (normal 15) |
| Two-stage prothrombin, U/ml | 200 |
| Fibrinogen, mg% | 294 |
| PTT, sec | 64 (normal 60) |
| Platelet factor 3 availability | decreased |
| Platelet TGT | decreased |
| After frozen and thawed | normal |
| Euglobulin lysis time, min | 160 |
| Γ VIII, % | 65 |
| Γ IX, % | 110 |
| Γ XIII | normal |

Table II ADP values in mg/3 × 10¹⁰ platelets are measured after incubation with collagen

| | Supernatant
PPP/mg | Platelets | Release of
ADP, % |
|---------|-----------------------|-----------|----------------------|
| Patient | 0.525 | 1.225 | 30 |
| Control | 1.470 | 0.720 | 67 |

there was an irregularity of joint surfaces and narrowing of the joint spaces. Examination of the spine revealed kyphoscoliosis and a marked osteoporosis.

Routine laboratory findings were within normal limits.

Laboratory investigations regarding bleeding tendency revealed a qualitative defect of platelets as summarized in table I. Bleeding time was slightly prolonged. Platelet factor 3 activity and availability were decreased, ADP (fig. 1) and distilled water induced primary aggregation normal (fig. 2) but there were no secondary aggregation curves. Collagen induced aggregation was slightly defective (fig. 3).

Total ADP content of platelets was normal but collagen induced ADP release decreased significantly (table II).

Discussion

A platelet defect exists in our case where the secondary aggregation related to normal release phenomenon does not occur and where the colla-

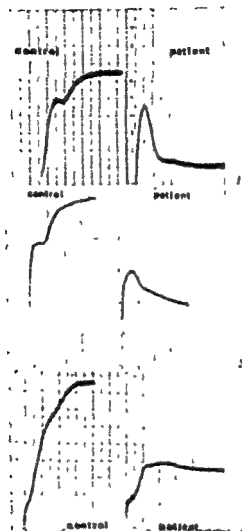


Fig. 1 Platelet aggregation induced by $0.5 \mu\text{g}$ ADP/ml

Fig. 2 Platelet aggregation induced by $2 \mu\text{g}$ red water

Fig. 3 Platelet aggregation induced by collagen

gen induced aggregation is defective. Collagen induced ADP and platelet factor 3 release were also defective. The platelet defect of this case is similar to the findings in primary thrombopathy [25-26]. In other words in this case an Ehlers Danlos syndrome and primary thrombopathy exist together.

Although the existence of bleeding diathesis in Ehlers Danlos disease is a known fact there is very little literature dealing with the platelet defect in this kind of cases. GOODMAN *et al* [6] observed in a family with Ehlers Danlos syndrome the prolongation of bleeding time, the defect in prothrombin consumption related to platelet factor 3 deficiency and the existence of defective clot retraction. In a case of Ehlers Danlos syndrome KASHIWABE *et al* [13] found similar results with platelet factor 3 release defect and ultrastructural findings similar to primary thrombopathy.

In our case we have found in the platelets a defective release of ADP and factor 3 in absence of secondary curve with ADP, adrenalin and distilled water and a defective curve with collagen which are similar to the findings in cases of primary thrombopathy [25-26].

The primary thrombopathy is characterized by a defect in the platelet release mechanism described in 1956 by FULTON and KARACA [29-30]. The authors drew attention to the fact that in some cases of primary thrombopathy several other congenital defects could exist together [27].

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Material und Methode

100 periphere Lymphozyten einer 24-jährigen Rubiolerpatientin (Luxemburg 3 Tage zuvor) wurden elektronenmikroskopisch ausgewertet [4]. Die Rubiolerkrankung nahm einen normalen klinischen Verlauf. Insbesondere ergab sich kein Hinweis für eine maligne Erkrankung des lymphatischen Systems. Gleiches gilt auch für andere Patienten mit verschiedenen Virusinfekten. Immunzytologische, autoradiographische und elektronenmikroskopische Untersuchungen durchgeführt. deren Methodik und Ergebnisse mitgeteilt wurden [1].

Befunde

54% der Lymphozyten der Patientin zeigten ein deutlich vermehrtes Ergastoplasma. Die Ergastoplasmazisternen und der perinukleäre Spalt eines Lymphozyten waren erweitert und enthielten lamellare Strukturen (Abb. 1, 2). Diese bestanden aus elektronendichten, weniger als 100 Å dicken Lamellen, die in einer Periodik von etwa 150 Å aneinandergelagert die Ergastoplasmazisternen sowie den perinukleären Spalt ausfüllten. Die Golgi-Zisternen waren frei von diesen Strukturen.

Diskussion

Die von uns beobachteten Strukturen entsprechende Einlagerungen wurden unseres Wissens bisher nicht beobachtet. Sie unterscheiden sich eindeutig von tubulären Zytoplasmainschlüssen normaler Lymphozyten [5] sowie von kristallinen Granula bei lymphoretikulären Systemerkrankungen [2], Langethans-Granula bei malignen Histiozytosen [6, 8] sowie ähnliche Einschlüsse bei leukämischer Retikuloendotheliose [9] und ebenfalls nicht mit ihnen zu verwechseln. Verdoppelungen der Ergastoplasmalamellen, die entfernt den von uns beobachteten Strukturen ähneln wurden bei lymphoretikulären Systemerkrankungen speziell [3, 7]

Abb. 1. Lymphozyt mit lamellären Einschlüssen im perinukleären Spalt und Ergastoplasmazisternen. Im Kern grober Nukleolus. M = Mitochondrium. Golgi-Feld = 11000.

Abb. 2. Ausschnitt aus Abb. 1 zeigt Teil des Kern angrenzenden perinukleären Spalts und des Ergastoplasmazisternen. Lamellen sind weniger als 100 Å Abstand voneinander gelagert. Die Lamellen bestehen aus 150 Å = 50000.

Die Bedeutung der bei unserer Rotelpatientin beobachteten lamellären, im Ergastoplasma und perinukleären Spalt lokalisierten Einschlüsse ist nicht bekannt. Sie demonstrieren die Variationsfähigkeit nichtmaligner Lymphozyten und reichen deutlich, dass morphologische Befunde bei malignen Erkrankungen vorsichtig zu interpretieren sind.

Zusammenfassung

In den Ergastoplasmazisternen und im perinukleären Spalt eines Lymphozyten einer Rotelpatientin wurden neuartige lamellare Einschlüsse beobachtet.

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Pyknotosis in Heat-Stroke

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Abstract. Large numbers of pyknotocytes were observed in the peripheral blood of a Negro gold miner shortly after he developed heat stroke. Examination of these cells under the scanning electron microscope showed aggregation of the cell content to one pole and fragmentation of the surface membrane. Heat stroke was complicated by uraemia, anaemia and mild hepatocellular damage. Attempts to induce this morphological change *in vitro* by heating erythrocytes for varying times at different pH's and in uraemic serum were unsuccessful. The mechanism of production of pyknotocytes in this patient is not known.

Key Words:
Erythrocytes
heating *in vitro*
Heat stroke
Pyknotocytes
Scanning
electron microscope

The effect of heat on erythrocytes has been studied both *in vivo* and, to a lesser extent, *in vitro*. At temperatures in the biological range of fever, increased osmotic and mechanical fragility occurs, with spontaneous haemolysis and shortened red cell survival [1, 4, 9-10]. At temperatures greater than 47° C, erythrocytes show additional striking and irreversible morphological changes, especially the formation of spherocytes and microspherocytes [6]. Similar changes have been demonstrated in patients with thermal burns [4]. Perhaps the clinical condition in which the adverse effects of heat on man can best be assessed is heat stroke. Although the changes in certain organs have been documented, little is known about the effect of heat stroke on erythrocytes. We report here a patient in whom pyknotocytes and haemolysis were demonstrated following such an episode, and describe our attempts to reproduce this morphological

change *in vitro*. The opportunity was taken to examine pyknotocytes under the scanning electron microscope and the appearances are described.

Case Report Including Special Investigations

A N = 25 year-old Negro from Malawi had been completely well before starting work on the gold mines. He was fully acclimatised and had shown no evidence of heat intolerance during the 10 months he had been employed. On 11th December 1971 after working for 4 h at a dry bulb temperature of 33.5 °C (92.3 °F), wet bulb temperature of 31.0 °C (87.9 °F) with a wind velocity of 0.45 mps and a wet bulb reading of 9.6 units, he was seen to be confused, mumbling and behaving inappropriately. His skin was hot and dry and his eyes were staring. Although his temperature was not taken a provisional diagnosis of heat stroke was made and cooling was commenced using a mixture of cold water and compressed air. He became violent during the procedure and then lapsed into coma. Cooling was continued for 1 h. He was then brought to the surface and admitted to the mine hospital.

On arrival the patient's blood pressure was 140/50 mm Hg, pulse 160 beats per minute and rectal temperature 42.3 °C (108.0 °F). The skin was dry but there was no obvious dehydration. The patient was comatose and swaying movements of the eyes were present. Intermittent convulsions occurred for the first 24 h. The heart and lungs were normal and the abdomen was slightly distended. The urine contained protein, cells and casts. The blood urea level was 65 mg/100 ml, serum sodium 140 mEq/l, potassium 2.6 mEq/l, chloride 116 mEq/l, carbon dioxide content 13.6 mEq/l and blood sugar concentration 135 mg/100 ml. The serum glutamic oxalacetic transaminase was 260 Reitman Frankel units, glutamic pyruvic transaminase 130 Reitman Frankel units and lactic dehydrogenase (LDH) 310 Wroblewski La Due units. Cooling was commenced and after 2.5 h the rectal temperature had fallen to 36 °C (96.8 °F). The patient was given 10 mg of diazepam and 40 mg of chlorpromazine by intramuscular injection and 500 mg of hydrocortisone intravenously. Intravenous fluids were administered. On the third day of illness the patient was transferred to the Johannesburg General Hospital for further management. At this time the rectal temperature was 39 °C (102.2 °F). He was semi-comatose and had pin-point pupils. There was no other obvious neurological deficit and the remainder of the examination was unremarkable.

The haemoglobin level was 14.5 g/100 ml, haematocrit 47%, erythrocyte count 4.6 million/mm³ and platelets 40 000/mm³. The leucocyte count was 11 600/mm³ with a differential count of 82% neutrophils, 2% eosinophils and 16% lymphocytes. Examination of a blood smear showed severe anisopoikilocytosis with numerous pyknotocytes (48%) (fig. 1). Coagulation studies revealed a prothrombin time of 12.5 sec, a normal kaolin partial thromboplastin time, fibrinogen 315 mg/100 ml, factor VIII assay 142% and factor V assay 110%. Fibrinogen degradation products were slightly increased at a level of 25.6 µg/ml (normal 4.10 µg/ml). The blood urea level was 130 mg/100 ml and the serum electrolytes were normal. The blood urea concentration remained at approximately the same level for 5 days before rising sharply to 319 mg/100 ml. The serum bilirubin concentration was

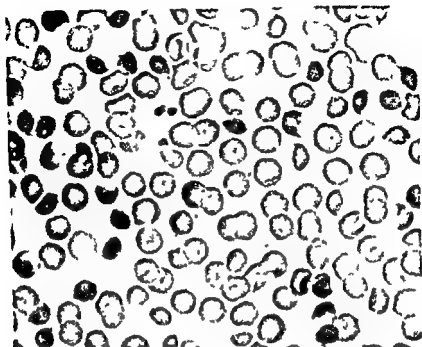


Fig. 1. Peripheral blood smear showing the presence of many pyknotocytes.

4.9 mg/100 ml on admission, reached a maximum of 6.6 mg/100 ml the following day and decreased thereafter, reaching 2.4 mg/100 ml on the tenth day.

Both urebilin and bilirubin were present in the urine during the first day. Subsequent haematological studies showed little alteration in the haemoglobin level but the leucocyte count rose to 17,600/mm³. Reticulocyte levels were markedly increased, rising to 8.0% 6 days after admission. Thrombocyte counts persisted at the original levels. Plasma haemoglobin levels were raised at 20.8 mg/100 ml (normal 2-7 mg/100 ml) in association with persistent haemoglobinuria. The number of pyknotocytes decreased progressively and when blood was taken for the last time on the tenth day constituted 1% of the peripheral smear. Bone marrow aspiration on the eighth day showed a normal cellular marrow. Erythropoiesis was normoblastic and of slightly decreased activity. Granulopoiesis was normal. The myeloid erythroid ratio was 4:1. Megakaryocytes were present in a slightly increased number.

Studies were carried out to exclude a possible haemolytic aetiology. The haemoglobin electrophoresis pattern was normal as were HbS and HbA₂ levels (1.0 and 1.9% respectively). An unstable haemoglobin was not demonstrated. The blood urea nitrogen level for plasma aphaeresis and nitrogenous derivatives done on the eighth day revealed normal urea levels, decreasing with a 43% decrease (normal 83-170 mg/dl).



Fig 2 Scanning electron photomicrograph showing a number of pyknotocytes as well as normal and mildly distorted cells. In one cell the surface membrane appears to lie in folds over the haemoglobin precipitate $\times 10,000$

Pyknotocytes were examined under the scanning electron microscope. Fresh blood from the patient was mixed with glutaraldehyde for fixation, the cells were packed by centrifugation, a thin smear made on a cover slip and the specimen air dried. Gold was evaporated onto the specimen in an AF112 model vacuum coating unit to approximately 1000 Å in depth. The cells were then examined using a Cambridge Stereoscan Mark 2A scanning electron microscope. The appearances are shown in figures 2-4.

The patient never regained consciousness and an electroencephalogram done on the fifth day showed a grossly abnormal tracing with virtual absence of any rhythmic activity. On the tenth day peritoneal dialysis was commenced but the patient died the following day. At necropsy the brain was oedematous and congested with petechial haemorrhages throughout, including the pons. The kidneys were pale and swollen and acute tubular necrosis was present. There was no obvious liver damage.

Because pyknotocytosis has not previously been reported either in heat stroke or on heating erythrocytes, we attempted to induce this change *in vitro*. Blood was collected from a healthy donor into ACD (0.50 g citric acid, 1.38 g sodium citrate and 1.39 g dextrose per 100 ml). The cells were washed free of plasma and buffy layer



Fig 3 Scanning electron photomicrograph of an erythrocyte in which the haemoglobin appears to have aggregated at one pole of the cell. It is uncertain whether the small shadows seen in the 'empty' part of the cell are membrane folds or small amounts of remnant haemoglobin. Projections of the cell membranes are clearly seen. (4000 \times)

Fig 4 Scanning electron photomicrograph of a grossly distorted cell with haemoglobin precipitated in one pole. The cell membrane appears to be distorted most in one area. (4000 \times)

by suspension in phosphate buffered saline (PBS), repeated centrifugation, removal of supernatant and resuspension in PBS. The washed cells were packed and aliquots suspended in equal volumes of PBS at the required pH. They were then incubated for 1, 4 or 6 h at temperatures of 37, 41, 44 and 46 $^{\circ}$ C while shaking with glass beads. The pH of the PBS was varied between 7.0, 7.2, 7.4 and 7.6. In those instances where whole blood was used, the pH of the suspension was changed to that required by using sodium α -PBS; the time of incubation and glass bead shaking was identical to that used with the washed cells. One further experiment was carried out where cells obtained from a patient with chronic renal failure (plasma creatinine 120 mg/100 ml) were washed as described before incubating them in the patient's own plasma at 37, 41 and 44 $^{\circ}$ C, at 7.4 and pH (6.9). At these temperatures and pH values no pyknocytes were seen in normal red cells were not incubated with the patient's serum.

Discussion

Haematological parameters have been variable when measured shortly after an episode of heat stroke [2, 3, 11]. This is probably due to a variety of factors, but particularly the state of the patient's hydration and per-

pheral circulation, and the circumstances under which heat-stroke occurred. What happens thereafter has been the subject of little systematic investigation. In an abstract published in 1955, HALDEFN *et al* [5] reported 15 patients in whom the mean haemoglobin level fell during the week following the onset of heat-stroke. There was no obvious blood loss and after carrying out red cell survival studies they concluded that anaemia was due to two factors, increased destruction of erythrocytes and failure of the bone marrow to respond adequately to the falling haemoglobin level. STEPHANINI and SPICER [13] subsequently reported one patient with haemolytic anaemia which was associated with increased fibrinolysis.

Pyknocytosis has not previously been reported in heat stroke. Pyknocytes or 'blister cells' are erythrocytes in which the haemoglobin appears to have aggregated at one pole of the cell leaving the other 'empty', an appearance which has been likened to that of a basket with a handle [8]. Pyknocytosis is rare and has only been described in association with the haemolytic phase of glucose-6 phosphate dehydrogenase (G-6-PD) deficiency [7] and sickle cell disease complicated by pulmonary infarction [8].

It is thus a phenomenon which has previously been reported only in erythrocytes which are intrinsically abnormal. In our patient haemoglobinopathy was excluded. Although the Motulsky screening test for G-6-PD deficiency was normal, this test may be unreliable during acute haemolytic episodes [12], so that G-6-PD deficiency or some other form of intrinsic abnormality may have remained undetected. That this is likely is suggested by the fact that pyknocytosis has not been produced in the many studies, including the present, in which erythrocytes have been heated *in vitro*.

The alternative possibility that pyknocytosis was induced by the combination of heat and some substance in the plasma seems less likely. The metabolic disturbances in our patient were similar to those commonly observed in heat-stroke and pyknocytosis has not previously been reported in heat stroke or induced in experimental animals [9] or human volunteers [1] by subjecting them to high temperatures. The most obvious metabolic change in our patient shortly after the onset of heat-stroke was a metabolic acidosis. However heating erythrocytes *in vitro* at varying pHs failed to reproduce the change. Repeating the experiment with uraemic serum was also unsuccessful.

The difficulty in understanding the mechanism of pyknocyte formation is compounded by uncertainty over the pathogenesis of heat injury to red

cells. In this regard, postulates have varied from inactivation of key enzymes to alterations of membrane structure and function [10]. It seems likely that pyknocyte formation represents a physicochemical alteration of the haemoglobin component of the erythrocyte. That there may also be an effect on the cell membrane is suggested by the scanning electron micrographs which show surface membrane alterations and projections.

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Mechanism of Action of L-Asparaginase on the Cell Cycle and Growth in Acute Lymphoblastic Leukemia¹

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Abstract. The effect of L-asparaginase on various acute human lymphoblastic leukemia cell populations was evaluated in an investigation of its mechanism of action with respect to the blast cell cycle and the growth of the leukemia cell population. Immediately following the injection of the drug, the number of cells in S increased while those in G₁ and M decreased. The percentage in G₁ showed no appreciable change. Later a decrease of the effect of L-asparaginase on cells in S phase was noted, together with reduction of the size of the proliferating compartment. Reference is made to recently proposed proliferation kinetic models in acute leukemia in support of the view that the action of the drug may also be directed towards cells in transition from G₁ to S.

Key Words:
Asparaginase
Autoradiography
Cell cycle
Cytospectrophotometry
Leukemia therapy
Lymphoblastic leukemia

L-Asparaginase is widely employed in human tumor therapy, particularly in the management of acute leukemias [8, 11, 21, 30, 31]. Its effect on the proliferative kinetics of these forms, on the other hand, has not yet been determined. It was therefore decided to examine this question with respect to certain parameters in acute lymphoblastic leukemia, since this form responds best to treatment with the enzyme. Two main questions were investigated: "At what point of the mitotic cycle do the earliest kinetic changes take place?" "How are the gradual reduction in size of the cell population and the loss of its proliferative activity brought about?"

Materials and Methods

Five cases of acute lymphoblastic leukemia were studied. Four had received no previous antileukemic therapy, one patient was in relapse 15 days after completion of standard

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methotrexate, vincristine and prednisone management. Peripheral white cell values ranged from 40 000 to 750 000/mm³ (lymphoblasts 84-98%). Secondary anemia, adenopathies, and/or more or less pronounced liver and spleen enlargement were constant. Hemorrhagic diathesis and oropharyngeal ulcers and necroses were occasional findings. Each patient received a single dose of 100-200 U/kg asparaginase (Crasmatin, Bayer) per day. The administration pattern is shown in figure 1. Marrow samples were taken by means of a normal technique and passed through thin needles: the disaggregated cells were counted. The following marrow and/or peripheral blood parameters were determined prior to and at various times after the commencement of the treatment: cell counts per mm³, percentage lymphoblasts, mitotic index (on at least 3 000 cells), mean lymphoblast diameter. On each occasion an aliquot was diluted with Hank's solution (2:1 or 1:1 according to cellularity) and incubated with 1-2.5 μ Ci/ml ³H-thymidine (specific activity 10 Ci/mM) for 30 min at 37 °C. After fixing in Carnoy's fluid the smears were covered with Ilford K₂ emulsion and exposed for 10-15 days at 4 °C. Development and fixing of the slides was followed by staining with Giemsa buffered to pH 7.4.

Labelling index (LI) and mean grain count (m.g.c.) values were determined on at least 3 000 cells and 100-200 cells respectively.

A microspectrophotometric method was used to evaluate cell DNA contents in cases 1, 3, 4 and 5, using photographic maps of Giemsa stained slides. Each cell photographed was assigned a number and its diameter was determined. After destaining the smears were subjected to Feulgen staining. DNA content values were then obtained for each numbered cell using a Barr and Stroud G-2 microdensitometer. The monochromatic light wavelength employed was ≤ 400 Å, and the values of absorbance were expressed in arbitrary units.

The slides were then autoradiographed as described and cells with a thymidine uptake were located on the maps. At least 400 cells were subjected to combined spectrophotometric and autoradiographic study on each occasion. Lastly, the method of Galosto *et al.* [17] was used to determine the growth fraction (GF) from the relationship between LI and cell diameter. With this method the size of the proliferating compartment is determined by simultaneous evaluation of the percentage of blast cells of different diameters and their respective labelling indices.

Results

Clinical and hematological response to treatment. A positive response was noted in all 5 cases. This included an improvement in the clinical picture with reductions in spleen and lymph node size and the disappearance of hemorrhages and fever. Changes in the blood picture were the first signs of the effectiveness of the treatment and included rapid falls in both peripheral and marrow lymphoblasts (fig. 1).

In cases 4 and 5, where frequent peripheral blood samples were obtained, blastis decreased after 6 and 7 h, respectively. As can be seen in figure 3 peripheral Ls were higher than those in bone marrow. This had

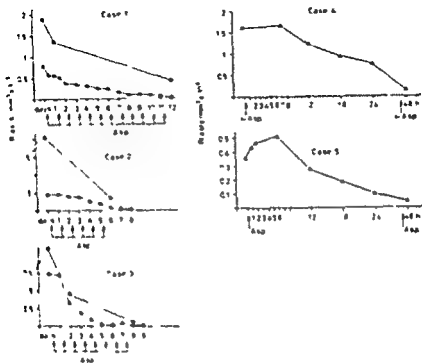


Fig. 1. Number of bone marrow and peripheral blasts before and during therapy with L-asparaginase. ● = Bone marrow blasts, ▲ = peripheral blasts.

been occasionally noted in infantile ALL [18, 19], as well as in many cases of differentiated lymphosarcomas, where LL, M1, G1, and large blast percents were higher in peripheral blood. This was therefore not considered as an end compartment, as usual, but as one formed by an actively proliferating lymphoblastic population. Cases 4 and 5 presented all these features, and the kinetic parameters for peripheral blasts were followed.

Fluctuation in LL, G1 and m.g.c. Marked falls in both peripheral and marrow LL values were noted 24 h after induction (Fig. 2 and 3). Cases 4 and 5 presented high peripheral LLs and frequent determinations showed an increase in the percentage of labeled cells soon after asparaginase, this was evident in case 4 though well marked and progressive to up to 3 times the initial value in case 5.

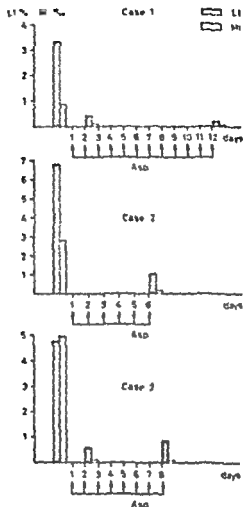


Fig 2 Bone marrow labelling index (LI) and mitotic index (MI) before and during therapy with L asparaginase in cases 1-3

MI values fell to almost zero within a few hours. When LI values increased, this decrease was noted very quickly. Mgc values were obtained in cases 4 and 5. Gradual reduction took place.

Changes in lymphoblast diameter showed that decreased LI values were accompanied by a decrease in the number of larger cells, resulting in a population composed of small cells with a relatively restricted spread of diameter values. GF values decreased progressively, though an initial increase in LI was accompanied by higher GF values (table I).

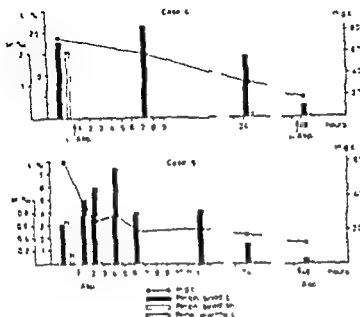


Fig. 3. Peripheral blood labelling index (LI), mitotic index (MI) and percentage of therapy bone marrow labelling index in cases 4 and 5.

Lymphoblast distribution in different phases of the cell cycle. Sequential determination of DNA spectrophotometric values and ^3H thymidine uptake was used to evaluate the distribution of lymphoblasts in different stages of the cell cycle prior to and during treatment. Figure 4 shows the DNA content of labelled and unlabelled blasts of case 4 while table I illustrates the cell distribution patterns in all cases. Before administration of the drug cells with diploid DNA were unlabelled and that represent the population fraction in G_1 or $G_0 + Z$. Apart from a small percentage in G_1 ($2-10\%$ with a DNA content of $2C$) all cells with greater than $2C$ DNA were labelled.

Cells with more than $2C$ DNA content decreased in number after the commencement of therapy in cases 1 and 3. In cases 4 and 5, on the other hand, early increases in the number of such cells paralleled the enhanced LI values coupled with percent reductions in cells in G_1 and M . These findings are seen as an expression of the accumulation of cells in S and a

Table 1. Distribution of blasts in the phases of cell cycle and growth fraction (GF) before and during therapy (values in %)

| Experimental time | Actually proliferating blasts | | | | | Non prol ferating blasts
(G ₀ + Z) = (1-GF) | GF ¹ |
|----------------------|-------------------------------|-----|----------------|-----|----|---|-----------------|
| | G ₁ | S | G ₂ | M | U | | |
| <i>Case 1</i> | | | | | | | |
| Before therapy | 333 | 35 | 11 | 1 | 0 | 620 | 0.35 |
| 48 h after L-aspar | 93 | 2 | 5 | 0 | 20 | 590 | 0.12 |
| <i>Case 3</i> | | | | | | | |
| Before therapy | 313 | 47 | 10 | 5 | 0 | 625 | 0.37 |
| 48 h after L-aspar | 134 | 5 | 4 | 0 | 1 | 856 | 0.14 |
| 8 days after L-aspar | 3 | 10 | 5 | 0 | 2 | 980 | 0.02 |
| <i>Case 4</i> | | | | | | | |
| Before therapy | 429 | 184 | 23 | 2 | 2 | 360 | 0.64 |
| 7 h after L-aspar | 463 | 220 | 6.5 | 0.5 | 0 | 310 | 0.69 |
| 24 h after L-aspar | 474 | 155 | 9 | 0 | 2 | 360 | 0.64 |
| 48 h after L-aspar | 235 | 16 | 10 | 0 | 9 | 730 | 0.27 |
| <i>Case 5</i> | | | | | | | |
| Before therapy | 348 | 25 | 6 | 1 | 0 | 620 | 0.35 |
| 4 h after L-aspar | 349 | 73 | 7 | 0 | 1 | 570 | 0.43 |
| 24 h after L-aspar | 180 | 17 | 10 | 0 | 23 | 770 | 0.23 |

Abbreviations: G₁ = unlabelled cells with 2C DNA content, S = ³H thymidine labelled cells with greater than 2C DNA content, G₂ = unlabelled cells with 4C DNA, M = mitotic index, U = unlabelled cells with DNA content between 2C and 4C.

¹ GF is expressed as a fraction of 1

slowing down of the passage of cells through this phase. After the 6th and 24th h, in fact, the number of cells in S (>2C DNA) fell to about 1%.

Unlabelled cells with DNA contents of over 2C and less than 4C (U cells) appeared at the 48th h in cases 1 and 4, and at the 24th h in case 5. These cells may be viewed as blocked in S phase.

Discussion

In acute lymphoblastic leukemia treated with L-asparaginase, the drug brings about changes in certain kinetic parameters and a redistribution of blasts throughout the different stages of the cycle at a very early stage, before any reduction in cell numbers takes place.

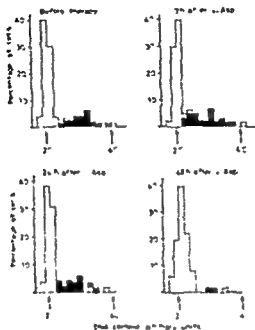


Fig. 4. DNA content of labelled and unlabelled blasts in case 4 before and during therapy.

A gradual increase in ^3H thymidine LI values was noted within the first 4-7 h in 8 cases. The mitotic index showed a marked downward course. Cells with more than 2C DNA displayed a percent increase parallel with that in the LI, accompanied by a fall in the number of unlabelled 4C cells. GI values were virtually unaffected. These data were used to calculate the percent distribution of the proliferating population over the different phases of the cell cycle. Immediately following the injection, the number of cells in S increased, while those in G_1 and M decreased. The percentage in G_1 showed no appreciable change.

L-Asparaginase seems to be responsible for a very prompt slowing down of the passage of cells through the S phase, coupled with their accumulation and blocked exit from the S phase. The fact that the passage from G_1 to S is not blocked in this stage can be deduced from the increase in LI values. The slowing down of the passage through S is also attested by the gradual reduction in m.p.c., in step with the increase in LI. This pro-

gressive fall-off in the uptake of ^3H -thymidine and its incorporation in DNA points to a decrease in the synthesis of DNA itself. A depression of DNA synthesis rate was already observed in 6C3HED lymphoma cultures added with L-asparaginase [6, 12].

An alternative explanation for the increase in LI values would be that L-asparaginase is directly responsible for damage to the already synthesized DNA chain, so that repair processes are at work at an early stage, before cell death occurs [13]. Yet this view is in conflict with the cytospectrophotometric demonstration of increased mean DNA contents running parallel with increase in LI values, and the uniform distribution of labelled cells between 2C and 4C DNA. By the same token, there is no sign of a DNA repair peak in the grain count curves.

The 24th and 48th h specimens presented evidence of serious damage to the proliferative capacity of the blast cells. The labelling data made it clear that there had been a marked fall in the percentage of cells capable of DNA synthesis. A 7- to 50 fold drop in LI values was coupled with a fall in the number of cells with greater than 2C DNA. GI values were much reduced and the MI was 0 in all cases.

In case 5, these changes were already apparent at the 6th h and thereafter became increasingly evident. When the first signs of clinical remission appeared at these later experimental times, there was a marked reduction in the leukemic cell population in all cases. Both marrow and peripheral cellularity were diminished and there was a fall in blast percents.

The residual population was homogeneous in a number of respects: diameters were small and lay within a narrow range, DNA values were 2C in nearly all cells. This means that nearly all the remaining cells probably are in a resting stage.

The effect of a single high dose of L-asparaginase (1,000-2,000 U/kg) has been studied by LAMPIN *et al* [24] and SAUNDERS [28] in 1 and 5 cases, respectively. LAMPIN noted a slight increase in LI after 1 h and a marked fall after 24 h, whereas MI values fell from the 1st h onwards. SAUNDERS observed a constant fall in LI values from the 6th h on, while MI values fell both later and more slowly. Attention must be drawn to the fact that SAUNDERS did not take the immediate post-injection picture into account. A further explanation for the discrepancy between his results and our cases 4 and 5 may be seen in his employment of a 20 fold higher dose of L-asparaginase in a single injection. We observed a cytolytic effect at a later period and it is at this point that the discrepancy between the two sets of data disappears. It may be suggested that the lower asparaginase doses, extensively employed for treatment, permit the detection of

continuously pass from G_0 to feed the proliferating compartment are activated.

Asparaginase-induced inhibition of the passage from G_0 to cycle has been demonstrated in two experimental models (blastogenesis of human lymphocytes [1, 26, 29], liver regeneration in the partially hepatectomized rat [2-4]). Our data suggest that asparaginase most probably act also at this level in lymphoblastic leukemia. Several days after the commencement of treatment morphologically and cytochemically homogeneous lymphoblasts were still present in considerable number. It can hardly be suggested that these were only cells in Z phase, since earlier works has shown that survival times in this compartment are of the order of no more than a few days [15, 22]. One can therefore suppose that these, too, were blasts in G_0 phase whose reentry into the proliferating compartment was being markedly inhibited, as was clear from the extremely low LI and GI values.

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The Effect of L-Asparaginase on DNA and RNA Synthesis by Lymphoblasts of Acute Lymphocytic Leukemia¹

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Abstract. Bone marrow cells of 8 patients with acute lymphocytic leukemia were studied by cytochemical and autoradiographic methods for the synthesis of nucleic acids in the presence or absence of L-asparaginase. The cultures included thymidine for DNA metabolism and RNA metabolism. In the absence of the enzyme the leukemic lymphoblasts reacted more intensively for DNA and RNA synthesis. The addition of the enzyme revealed depressed incorporation of the labeled precursors by the putative cells.

Key Words:
Asparaginase
Bone marrow culture
Cytochemistry
Autoradiography
DNA and RNA synthesis
Lymphocytic leukemia

L-Asparaginase is a known inhibitor of the blast transformation of lymphocytes *in vivo* as well as *in vitro* [2, 9]; it appears to exert its effects by depressing susceptible cells of exogenous L-asparagine which results in the inhibition of protein synthesis [1, 3-4]. Associated with the inhibition of protein synthesis is the repression of nucleic acid metabolism as shown by experiments with some animal tumors [6].

In the present investigation the effect of L-asparaginase on nucleic acid synthesis was studied cytochemically [5-8] to characterize the intensity of deoxythymine acid (DNA) and ribothymine acid (RNA) reactions. Moreover, to evaluate the inhibitory activity of L-asparaginase in DNA and RNA metabolism, the percentage of large blast cells incorporating thymidine and uridine in the presence or absence of the enzyme was determined.

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In the present investigation the effect of L-asparaginase on nucleic acid synthesis was studied cytochemically [5, 8] to characterize the intensity of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) reacting. Moreover, to evaluate the inhibitory activity of L-asparaginase in DNA and RNA metabolism, the percentage of large nucleic acids incorporating thymidine and uridine in the presence or absence of the enzyme was determined.

Materials and Methods

Bone marrow cultures of 8 patients with acute lymphocytic leukemia (ALL) were prepared and included Spinner modified Eagle's minimum essential medium supplemented with L-glutamine, fetal calf serum, phytohemagglutinin, and L-asparaginase. Cultures without the enzyme were also processed. The cultures were incubated with the addition of thymidine or uridine. The epon embedded specimens were dipped in Kodak NTB₂ and developed with Kodak D19_a developer. The slides were stained with Giemsa stain and evaluated by counting 300 large lymphoblasts from each specimen for heavily labeled, lightly labeled, and nonreactive cells.

Results

The primitive cells of ALL stained by the Feulgen reaction for DNA tended to stain more intensely with many cells possessing discernable, larger aggregates of chromosomal bodies than similarly stained cells incubated in cultures containing L-asparaginase (fig. 1a, b). Likewise, the primitive cells of ALL following the methyl-green-pyronin method for RNA appeared to be more reactive than leukemic lymphoblasts cultured in the presence of L-asparaginase prior to staining (fig. 1c, d).

Thymidine labeling for DNA synthesis showed many of the leukemic lymphoblasts to be heavily labeled with fewer nonreactive cells; similar specimens treated with L-asparaginase and thymidine demonstrated more nonreactive lymphoblasts and fewer lightly and intensely labeling cells (table I, fig. 2a, b). RNA metabolism using uridine in the presence of L-asparaginase revealed depressed incorporation of the label into the leukemic lymphoblasts with more cells appearing nonreactive and fewer cells showing intense reactivity. In comparison, without the enzyme, cellular incorporation of uridine exhibited many heavily and lightly labeling cells with fewer nonreactive lymphoblasts (table I, fig. 2c, d). With both thymidine and uridine, background labeling was light.

Discussion

L-Asparaginase is used as a chemotherapeutic agent in acute leukemias and malignant animal and human tumors because of its antitumor activity. The cells in various tumors are found to be asparaginase-sensitive or asparaginase-resistant. Asparaginase-resistant cells have high levels of the enzyme, asparagine synthetase, and have the ability of making asparagine

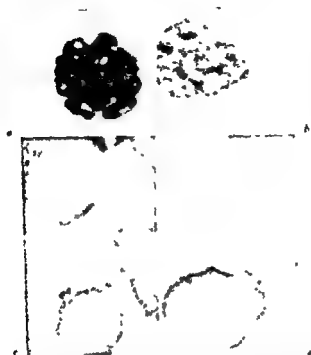


Fig. 1. Primitive cells of acute lymphocytic leukemia. Feulgen reaction. *a* Intense nuclear activity with visible aggregates of chromosomal bodies in a lymphoblast from cultures without L-asparaginase. *b* A few visible chromatin granules in a lymphoblast cultured with L-asparaginase. Methylgreen-erythrosin staining. *c* Intense cytoplasmic reaction in lymphoblasts from cultures without L-asparaginase. *d* Light cytoplasmic reaction in lymphoblasts incubated with L-asparaginase. $\times 1,500$.

Table I. The incorporation of thymidine and uridine by lymphoblasts of acute lymphocytic leukemia cultured in the absence or presence of L-asparaginase and expressed as the average per centage of $3H/cell$.

| | Without L-asparaginase | | | With L-asparaginase | | |
|-----------|------------------------|-----------------|---------------|---------------------|-----------------|---------------|
| | Mean % labeled | Light % labeled | non-trans. or | Mean % labeled | Light % labeled | non-trans. or |
| Thymidine | 41.9 | 99.0 | 19.1 | 2.2 | 29.9 | 9.1 |
| Uridine | 95.2 | 91.7 | 9.1 | 2.6 | 27.7 | 49.7 |

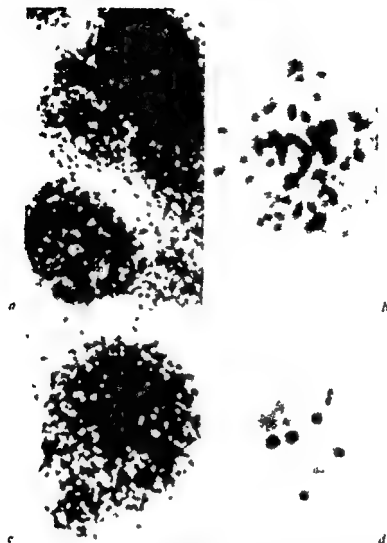


Fig. 2 Primitive cells of acute lymphocytic leukemia. Thymidine labeling: a) Heavier labeling in lymphoblasts from cultures without L-asparaginase; b) Lighter labeling in a lymphoblast cultured with L-asparaginase. Uridine labeling: c) Heavier labeling in a lymphoblast incubated without L-asparaginase; d) Lighter labeling in a lymphoblast cultured with L-asparaginase. $\times 1,500$

in sufficient quantities for their own metabolic requirements [11]. The sensitive cells are deficient in asparagine synthetase [7, 11, 13] and, when deprived of asparagine by L-asparaginase, show a depression of protein metabolism since they cannot synthesize adequate amounts of the amino acid. For growth, the sensitive cells must depend on the asparagine present

in the blood which in normal human plasma is reported to be 0.5-0.7 mg/100 ml [9]. Likewise, in order to grow in culture sensitive cells require the addition of L-asparagine to the culture media.

A recent investigation indicates that Jensen sarcoma cells in the presence of L-asparaginase are repressed in their synthesis of DNA probably resulting from the depression of protein synthesis which is considered essential for the initiation of DNA metabolism [10]. Once initiated protein synthesis may or may not be required to maintain DNA synthesis [12]. Mouse lymphomas depleted of asparagine by L-asparaginase demonstrate the inhibition of all types of RNA. The depletion of the amino acid may cause the selective inhibition of the synthesis of ribosomal RNA followed by the depression of messenger RNA and finally transfer RNA metabolism [6].

In the present study the repression of DNA and RNA synthesis in the primitive cells of ALL, evaluated cytochemically and by the incorporation of labeled precursors in the presence of L-asparaginase, tends to agree with the results of these investigations [6, 10, 12]. In cultures with L-asparaginase, the disappearance of heavily labeled cells points to the decreased rate of DNA and RNA synthesis, suggesting that the effect of L-asparaginase on DNA and RNA synthesis is secondary to the repression of protein synthesis.

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Lymphocyte Surface Markers in Lymphoproliferative Disorders

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Abstract. Peripheral blood lymphocytes of 10 normal controls and 32 patients with lymphoproliferative disorders were examined for the presence of surface markers. T cell frequency was decreased in chronic lymphocytic leukemia (CLL), Hodgkin's disease and myeloma, and was normal or decreased in acute lymphocytic leukemia (ALL). Lymphocytes with surface membrane receptors specific for IgG were markedly increased in lymphosarcoma and cryoglobulinemia, whereas extremely decreased in ALL. Normal in Hodgkin's disease, only lymphocytes specific for IgM were increased in CLL. B-cells (T_H or C) were diminished or absent in CLL and ALL. In the remission phase of ALL, TAC and T_H c-cells returned to normal and sIg were almost normal. The approach presented in this article suggests a classification of these disorders in relation to the T and B cell frequency which may be useful for diagnosis and therapeutic implications.

Key Words:
TAC c-cells
T_H c-cells
Immunofluorescence
Immunoglobulins
Lymphoproliferative
diseases
Surface markers

Lymphocytes are divided into two major groups according to their origin and function. T lymphocytes derived from the thymus [3-16] are concerned with cellular immunity [14], and B lymphocytes derived from an unknown site but analogous to the bursa of Fabricius in birds are concerned with humoral antibody production [2-31]. These cells can further be differentiated according to specific surface markers which permit them to be related to cellular or humoral functions [9, 10, 16].

Surface markers are used to measure the frequency of T and B cells and have also been used to describe different stages of lymphoid cell differentiation [24, 25, 26]. B cells can be identified by the presence of membrane surface immunoglobulins (sIg) [11, 21, 27, 32], which bind specific heavy

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Abstract. Peripheral blood lymphocytes of 18 normal controls and 32 patients with lymphoproliferative disorders were examined for the presence of surface markers. T cell frequency was decreased in chronic lymphocytic leukemia (CLL). Hs antigen was absent in CLL, and was normal or decreased in acute lymphocytic leukemia (ALL). Lymphocytes with surface membrane receptors specific for a IgG were markedly increased in lymphosarcoma and crivomastocytoid leukemia, severely decreased in ALL, normal in CLL. Hs antigen of case early lymphocytes specific for IgM were increased in CLL. Receptors for C3 were diminished or absent in CLL and ALL. In the remission phase of ALL, TAC and T_H markers returned to normal and sIg were almost normal. The approach presented in this study suggests a classification of these disorders in relation to the T and B cell frequencies which may be useful for diagnosis and therapeutic implications.

Key Words:
TAC system
T_H system
Immunofluorescence
Immunoglobulin
Lymphocyte Leukemia
Hs antigen
Surface markers

Lymphocytes are divided into two major groups according to their origin and function. T lymphocytes derived from the thymus [1-16] are concerned with cell-mediated immunity [17], and B lymphocytes derived from an unknown site but homologous to the bursa of Fabricius in birds are concerned with humoral antibody production [7-31]. These cells can further be differentiated according to specific surface markers which permit them to be related to cell-mediated humoral functions [2-10, 18].

Surface markers are used to measure the frequency of T and B cells and have also been used to describe different stages of lymphoid cell differentiation [14, 20, 21]. B cells can be identified by the presence of membrane surface immunoglobulin (sIg) [11-21, 23, 25, 32], which bind specific heavy

chain antiserum, their characteristic receptor for C3 complement component [5, 8] and their ability to bind certain antigen-antibody complexes [4]. T cell frequency can be measured by their spontaneous formation of rosettes with sheep red blood cells [6, 13, 19] and more recently by their ability to react with specific human anti-T serum [2].

The present study is concerned with determining the frequency of T and B cells in lymphoproliferative diseases through the use of surface markers specific for B cells – sIg and receptors for C3-activated complement component (EAC test) – and T cells, the spontaneous formation of rosettes with sheep red blood cells (E test).

Materials and Methods

Patients Normal controls were laboratory personnel, medical students, and their respective relatives. All controls were examined according to accepted clinical and laboratory criteria including estimation of the immunoglobulin serum levels, and were found to be normal in every respect.

32 patients with lymphoproliferative disorders consisted of 3 treated and 7 untreated cases of chronic lymphocytic leukemia (CLL), 1 case of primitive cryoglobulinemia, 1 case of lymphosarcoma, 6 cases with Hodgkin's disease, 5 cases with multiple myeloma, 20 cases with benign monoclonal hypergammaglobulinemia and 7 cases with acute lymphocytic leukemia (ALL). Three of the patients with ALL were examined in the acute phase and had not received previous treatment, while the other 4 had been treated and were asymptomatic in remission. The diagnosis of the lymphoproliferative disorders was based on accepted clinical and laboratory criteria, including bone marrow and lymph node biopsies in all cases, and isolation and typing of the monoclonal components in the serum and urine in the cases of myeloma.

Lymphocyte preparations and immunofluorescent staining Lymphocytes were isolated on a Ficoll isopaque gradient [26], washed 3 times in gelatin veronal buffer (GVB), and stained by membrane immunofluorescence [14] by incubation with fluorescein-conjugated antisera specific for the different heavy chain immunoglobulin classes. An ultraviolet microscope with vertical illumination was used to identify cells positive for membrane immunofluorescence [23].

Test procedure for E rosettes The test for E rosettes was performed by incubating lymphocytes with sheep red blood cells (SRBC) for 15 min at 37°C, then overnight at 4°C [13]. 200 lymphocytes were counted, those binding more than 3 SRBC were considered positive.

Test procedure for EAC rosettes The test for EAC rosettes was performed according to JONDAIL *et al* [13]. 0.25 ml of SRBC previously treated with rabbit IgM anti SRBC and human complement were mixed with an equal volume of lymphocyte suspension in GVB and EDTA at 37°C for 15 min [23]. 200 lymphocytes were counted, those binding more than 3 SRBC were considered positive.



Fig. 1. IgG detected by immunofluorescence showing a characteristic uneven or granular fluorescence on the greater part of the cell surface.



Fig. 2. Fluorescence of lymphocytes with IgG molecules binding more than 1 SRBC.

Results

The positive cells observed by immunofluorescence showed a characteristic uneven or granular fluorescence on the greater part of the cell surface (Fig. 1). Negative cells showed no detectable fluorescence. Concerning the lymphocytes in the normal control, 4.72% reacted with the fluorescein-conjugated anti-serum specific IgG, 4.3% with anti-IgA, 3.9% with anti-IgM, 0.9% with anti-IgD, and 0.7% with anti-IgE (Table I). Cells were found for the formation of rosettes were considered positive with more than 1 SRBC on their membrane (Fig. 2). The percentage of normal lymphocytes forming IgG rosettes was 21.4, whereas 42.4% formed E rosettes (Table I).

Table I Surface markers on normal peripheral blood lymphocytes

| Subjects | Surface immunoglobulins ¹ | | | | | EAC ²
rosettes | E ³
rosettes |
|----------|--------------------------------------|------|------|------|------|------------------------------|----------------------------|
| | G | A | M | D | I | | |
| V.A | 7 | 3 | 8 | 0 | 0 | 24 | 46 |
| S.C | 6 | 2 | 7 | 0 | 0 | 34 | 69 |
| M.A | 12 | 4 | 16 | 1 | 4 | 13 | 50 |
| D.A.R | 10 | 6 | 6 | 0 | 0 | 30 | 50 |
| D.A.C | 8 | 6 | 6 | 0 | 0 | 18 | 54 |
| L.E | 4 | 6 | 10 | 2 | 1 | 30 | 47 |
| F.A | 8 | 8 | 12 | 1 | 2 | - | - |
| L.E | 2 | 4 | 8 | 1 | 0.5 | 21 | 61 |
| M.O | 12 | 8 | 10 | 3 | 0 | 22 | 40 |
| D.A | 8 | 2 | 4 | 5 | 2 | 22 | 43 |
| S.A | 3 | 4 | 8 | 1 | 0 | - | 30 |
| C.M.V | 6 | 2 | 8 | 0 | - | 21 | 57 |
| V.M | 5 | 4 | 5 | 0.5 | 0.5 | 25 | 40 |
| L.G | 12 | 4 | 12 | 0 | 0 | 13 | 57 |
| S.E | 7 | 1 | 10 | 0 | 0 | 23 | 50 |
| G.S | 12 | 4 | 12 | 0.5 | 0.5 | 23 | 53 |
| P.F.F | 4 | 8 | 10 | 0 | 0 | 26 | 43 |
| L.V | 3 | 1 | 9 | 1 | 1 | 23 | 52 |
| Mean | 7.2 | 4.3 | 8.9 | 0.9 | 0.7 | 23.4 | 52.4 |
| Range | 2-12 | 1-8 | 4-16 | 0-5 | 0-4 | 13-34 | 40-69 |
| SD | 3.27 | 2.29 | 1.17 | 1.00 | 1.00 | 5.74 | 7.25 |

¹ Values are expressed in percentage of the cells positive for membrane immunoglobulins

² EAC = lymphocytes which form rosettes with sheep red blood cells coated with anti-sheep rabbit erythrocyte of class IgM plus human complement

³ E = lymphocytes which spontaneously form rosettes with SRBC

The results pertaining to patients with lymphoproliferative disorders are reported in table II. In untreated CLL, sIg were normal for IgG and IgA and significantly increased for IgM and IgD. Only in 2 cases was there evidence of IgE. The formation of FAC and E rosettes was significantly decreased. In treated CLL, lymphocyte sIg were normal or decreased except for IgM which was increased, though not to the extent observed in the untreated cases. EAC and E rosettes were significantly decreased. In the cases of CLL complicated with agammaglobulinemia, the percentage of lymphocytes positive for sIg was decreased except in 1 case which had an increase in IgM and normal IgG. FAC rosettes were low and E rosettes normal. In

Table II. Surface markers on lymphocytes of patients with lymphoproliferative disorders

| Case No. | Diagnosis | Lymphocytes with surface Ig % | | | | | EAC rosette % | T rosette % |
|----------|-----------|-------------------------------|----|----|----|---|---------------|-------------|
| | | G | A | M | D | I | | |
| 1 | CLL u | 6 | 0 | 49 | 3 | 0 | 0 | 13 |
| 2 | CLL u | 8 | 6 | 51 | 3 | 0 | 1 | 11 |
| 3 | CLL u | 4 | 3 | 78 | 11 | 4 | 11 | 18 |
| 4 | CLL u | 8 | 4 | 92 | 6 | 2 | 6 | 11 |
| 5 | CLL u | 10 | 6 | 74 | 4 | 1 | 18 | 2 |
| 6 | CLL u | 2 | 6 | 12 | 0 | 0 | 5 | 24 |
| 7 | CLL u | 6 | 2 | 34 | 9 | 0 | 6 | 42 |
| 8 | CLL t | 2 | 8 | 41 | 0 | 0 | 9 | 34 |
| 9 | CLL u | 6 | 3 | 26 | 0 | 0 | 12 | 46 |
| 10 | CLL u | 2 | 0 | 0 | 0 | 0 | 1 | 49 |
| 11 | CMG | 12 | 20 | 66 | 4 | 0 | 20 | 55 |
| 12 | L | 1 | 16 | 14 | 2 | 4 | - | 44 |
| 13 | Hodgk | - | 8 | 2 | - | - | 27 | 46 |
| 14 | Hodgk | - | 2 | 10 | - | - | 30 | 34 |
| 15 | Hodgk | - | 0 | 0 | 0 | 0 | 2 | 34 |
| 16 | Hodgk | not | 2 | 6 | 0 | 0 | 14 | 39 |
| 17 | Hodgk | not | 2 | 4 | 2 | 0 | 16 | 32 |
| 18 | Hodgk | not | 2 | 10 | - | - | 30 | 34 |
| 19 | M IgG | - | 2 | 8 | 0 | 0 | 54 | 51 |
| 20 | M IgG | - | 4 | 2 | 0 | 0 | 45 | 24 |
| 21 | M IgG | - | 9 | 6 | 2 | 0 | 26 | 29 |
| 22 | M IgA | - | 11 | 0 | 0 | 0 | 29 | 32 |
| 23 | M IgA | - | 2 | 6 | 0 | 0 | - | - |
| 24 | BMIH | 1) | 2 | 6 | 0 | 0 | 39 | 73 |
| 25 | BMIH | 1) | 1 | 4 | 0 | 0 | 8 | 63 |
| 26 | ALL u | not | 1 | 3 | - | - | 12 | 31 |
| 27 | ALL u | not | - | 0 | 0 | 0 | 2 | 24 |
| 28 | ALL u | not | 3 | 0 | 0 | 0 | 8 | 92 |
| 29 | ALL u | not | 1 | 4 | - | - | 9 | 27 |
| 30 | ALL re | - | 1 | 4 | - | - | 27 | 88 |
| 31 | ALL re | - | 2 | 4 | - | - | 31 | 45 |
| 32 | ALL re | - | - | 1 | - | - | 24 | 63 |
| 33 | ALL re | - | 1 | 4 | - | - | 36 | 63 |

Abbreviations: CL
 Chronic lymphocytic
 leukemia; CMG
 Chronic monoclonal
 B-cell lymphoma;
 BMIH B-cell
 mixed lymphoma;
 ALL acute lymphocytic
 leukemia; ALL re
 relapsed ALL; ALL u
 undifferentiated ALL;
 Hodgk Hodgkin's
 disease; L lymphoma;
 M IgG monoclonal
 immunoglobulin G;
 M IgA monoclonal
 immunoglobulin A;
 not not tested.

1) tested with
 anti-CD3
 monoclonal antibody
 (OKT3).
 2) tested with
 anti-CD4
 monoclonal antibody
 (OKT4).

3) tested with anti-CD8
 monoclonal antibody
 (OKT8).
 4) tested with anti-CD16
 monoclonal antibody
 (OKT16).

the case with CMG, all sIg were increased. IgG slightly, and IgA, IgM and IgD markedly. The EAC and E tests were normal. In the case of lymphosarcoma, all sIg were significantly elevated. The EAC test was not performed and the E test was normal. In Hodgkin's disease, the sIg were normal except in 1 case which was reduced. EAC rosettes were normal except in case 15, and all had reduced E rosettes.

In multiple myeloma, sIg were normal or decreased, only case 22 showed complete absence of IgA and IgM. EAC tests were normal or increased and E tests were decreased. In benign monoclonal hypergammaglobulinemia, sIg for IgG and IgM were normal and IgA was decreased. EAC tests were normal in one and markedly reduced in the other, E tests were normal.

In untreated ALL, all sIg were extremely low. In case 28, IgG and IgA were absent and in case 27 all sIg were absent. The formation of EAC rosettes was very low and E rosettes were normal or decreased. In the patients in remission, sIg were similarly reduced, however, not to the extent found in the untreated cases. EAC and E tests were normal in 4 of the 5 cases. Peripheral blood smears of the patients in remission did not show any lymphoblasts or atypical cells.

Discussion

The analysis of sIg, EAC and E rosettes on peripheral blood lymphocytes of patients with lymphoproliferative disorders has provided us with information concerning the frequency of T and B cells. In comparing our results with those of others [1, 26] we found that sIg levels for IgM in untreated CLL are markedly increased, whereas in the treated cases and in the patients with associated γ gammaglobulinemia, IgM levels were only moderately increased, however, in comparison to the untreated, they are significantly reduced. The difference observed by others in similar patients is probably correlated with the number of peripheral blood lymphocytes or the stage of the disease or treatment [1, 20, 24, 26]. An established explanation as to the origin of the type B lymphocytes with sIg specific for IgM has not been provided. In cases 2-5 of untreated CLL, we measured EAC rosettes using mouse complement; the respective percentages were 28, 16, 5 and 20. There was a significant increase in the number of lymphocytes forming EAC rosettes in only one case. This is not in agreement with Pisci *et al* [22] who found high levels of EAC rosette formation with mouse complement in CLL. This discrepancy can be due to different technique, measurement of patients

in different stages of the disease or the different modulation patterns in the membrane of pathologic lymphocytes which produce different surface receptors for human and mouse complement. The low level of E rosettes is in agreement with the reduced cellular immunity in both *in vivo* and *in vitro* studies performed by others [7, 17].

In cryoglobulinemia and lymphosarcoma, there was an increase in lymphocytes specific for IgG, IgA and IgM. Present experience leaves us unable to come to a valid explanation as to their origin; however, we can conclude that there is a possible defect in maturation or an imbalance in Ig synthesis and secretion (synthesis being greater than secretion).

In Hodgkin's disease B cell frequency is normal whereas T cell frequency is decreased. This is in agreement with the defect in T cell function observed by others with *in vitro* PHA response [12]. It is interesting to note that in myeloma there is a partial defect in B lymphocytes related to sIg which is variable and not specific for the particular type of myeloma. These results agree with those of Coombs [personal communication] in 18 cases of myeloma. On the contrary, the lymphocytes forming EAC rosettes are increased which confirms the independence of two populations of lymphocytes or the possibility of the same cell having an increase in one or both receptors.

It is interesting to note the different patterns of lymphocytes with membrane receptors in the treated and untreated cases of chronic and acute leukemia. Lymphocytes in untreated cases of ALL lack surface Ig, have decreased EAC rosette and decreased or normal E rosette formation. This indicates that in some cases of ALL T cells are the dominant population while in others the lymphocytes belong neither to T nor B cell populations. In remission there is a low level of sIg and normal formation of EAC and E rosettes. This reversion toward normalcy indicates that it is possible to follow the course, measure the degree of severity and forecast the prognosis in these patients, and possibly in other lymphoproliferative disorders, by simply measuring the frequency of lymphocytes through the use of surface markers.

Lymphoproliferative diseases have individual characteristic patterns of lymphocytes with surface markers, and with a working knowledge of these patterns one would be able to confirm the clinical diagnosis and evaluate the severity of lymphoproliferative diseases. Further study at different stages of these diseases is at present desirable. With the identification of other markers, we foresee the possibility of using the identification of surface markers not only as a routine estimation of the early detection of cancer but also for the evaluation of the stage and the prognosis of the disease.

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Lymphoproliferative diseases have individual characteristic patterns of lymphocytes with surface markers, and with a working knowledge of these patterns one would be able to confirm the clinical diagnosis and evaluate the severity of lymphoproliferative diseases. Further study at different stages of these diseases is a present desiderata. With the identification of other markers, we foresee it possible to use the identification of surface markers not only as a tool for diagnosis but for the early detection of cancer, but also for the evaluation of response and the prognosis of the disease.

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Table I. Test subjects with different diseases

| Patient No. | Age, years | Sex | Diagnosis |
|-------------|------------|-----|--|
| 10 | 22 | w | epilepsy |
| 11 | 31 | w | epilepsy |
| 12 | 40 | w | epilepsy |
| 13 | 30 | w | hyperthyroidism gravid |
| 14 | 24 | w | hepatitis infectiosa |
| 15 | 63 | m | uremia gravid, degenerative polyneuritis |
| 16 | 31 | m | cirrhotic hepatitis |
| 17 | 22 | w | hyperparathyroidism malig. |
| 18 | 45 | w | melanoma malig. |
| 19 | 49 | w | rheumatoid arthritis |
| 20 | 19 | m | excessive intake of vitamin |

The table excludes Nos. 1-9 the young and healthy control subjects. Numbering of subjects III-29 is the same as in table III.

therefore, not suited for the study of extensive materials or for mass working.

After some initial technical difficulties we have been able to start collecting control material and, along with it, type cases from every pathological sector. Prior to the present paper, we have been able to show preliminarily that the protein binding of FAA in the course of normal pregnancy undergoes changes in that FAA binding to proteins, and primarily to transferrin, increases. At the same time the binding to α_2 -macroglobulin decreased. Very soon after delivery the FAA was found to have disappeared from the transferrin site (5).

The albumin variation of diphenylhydantoin (DPH) is well known to be connected with a fall in the FAA content of the serum. It has been assumed that the medication caused the FAA deficiency. To date it has not been possible to explain this phenomenon and it has been attributed to various factors, either to a disturbed absorption of FAA from the gastrointestinal tract (2). In the following we will describe the effect of DPH on the binding of FAA to serum proteins. At the same time findings concerning some normal diseases will be presented showing that many factors affect the FAA equilibria. It is especially in each case these changes are can probably be answered by large estimates that

Table II Protein and FAA distributions of the healthy control subjects in chromatography

| Protein zones | Mean | SD | Range |
|----------------------|-------|------|------------|
| Whole serum | | | |
| Proteins, mg/ml | 78.3 | 6.7 | 70.8-89.4 |
| FAA, ng/ml | 4.1 | 1.3 | 3.0-5.8 |
| FAA/protein, ng/g | 20.6 | 5.2 | 11.6-27.2 |
| Protein-bound FAA, % | 41.0 | 19.5 | 22.0-82.0 |
| Zone I | | | |
| Proteins, % | 13.0 | 2.8 | 10.0-17.0 |
| FAA, % | 3.0 | 2.6 | 0.0-8.0 |
| FAA/protein, pg/mg | 4.0 | 4.3 | 0.0-12.0 |
| Zone II | | | |
| Proteins, % | 7.0 | 1.4 | 5.0-9.0 |
| FAA, % | 21.0 | 8.4 | 10.0-34.0 |
| FAA/protein, pg/mg | 65.0 | 49.0 | 40.0-185.0 |
| Zone III | | | |
| Proteins, % | 2.0 | 1.3 | 1.0-4.0 |
| FAA, % | 12.0 | 3.0 | 8.0-17.0 |
| FAA/protein, pg/mg | 129.0 | 76.0 | 63.0-261.0 |
| Zone IV | | | |
| Proteins, % | 7.0 | 2.2 | 5.0-11.0 |
| FAA, % | 41.0 | 16.6 | 29.0-63.0 |
| FAA/protein, pg/mg | 122.0 | 35.8 | 72.0-167.0 |
| Zone V | | | |
| Proteins, % | 70.0 | 3.3 | 68.0-76.0 |
| FAA, % | 22.0 | 11.4 | 7.0-41.0 |
| FAA/protein, pg/mg | 6.0 | 3.3 | 3.0-13.0 |

The ordinal numbers I-V of the protein zones are the same as in figures 1-4

Material and Methods

The control series consisted of 9 healthy students and sportsmen, 7 men and 2 women, aged 15-26 (mean 23) years. The total results are shown in table II and the type chromatograms in figures 1 and 2.

The series of the patients studied consisted of 11 test subjects, with characteristics shown in table I. All patients were examined at the University Clinics by the relevant specialists. All the facilities afforded by clinical chemistry and a roentgen laboratory, and by serologic and cytologic methods were used in making the diagnosis. An effort was made to choose the present test subjects from among the

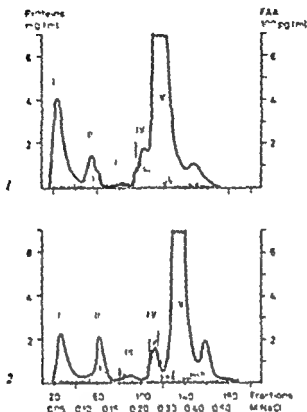


Fig 1 Typical picture of the chromatography of the serum of a healthy woman. Vertical lines indicate FAA. Protein zones are numbered from I to V.

Fig 2 Typical picture of the chromatography of a healthy man. Symbols are the same as for figure 1.

so-called type cases whose clinical diagnosis was clear cut and certain. At the time the specimen was taken for chromatography the patient had not yet received any treatment and was in his natural pathological state.

Specimens of venous blood were taken for DEAE Sephadex chromatography in the morning after an overnight fast. Blood samples for DPH determination were simultaneously taken from patients No. 10-12 (table I). The DPH contents were No. 10, 0.7 mg/100 ml; No. 11, 1.6 mg/100 ml; and No. 12, 2.6 mg/100 ml. All 3 patients had taken 400 mg DPH daily for more than a year.

The chromatography and associated laboratory procedures have been described in detail previously [9]. The FAA of the serum and the chromatography fractions was determined by the 1 case method [4].

The results of chromatography for the normal series are presented in table II and for the test series in table III. The type illustrations of the chromatograms of normal subjects are shown in figures 1 (woman) and 2 (man); those of the test patients in figures 3 (DPH user) and 4 (osteomyelitis).

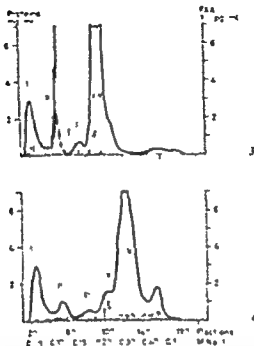


Fig. 3. Chromatography of the serum of a DPH user. Symbols as in Figure 1. The subject (No. 11 of table I) had 30 mg/day of DPH for 11 months. Serum DPH was 1.6 mg/100 ml. Note the increased FAA in the transferrin zone.

Fig. 4. Chromatography of the serum of a 1st year old patient with osteomyelitis. Symbols as in Figure 1. Note the decreased FAA in the pretransferrin zone.

Results

Table II shows the normal distribution of FAA in the chromatography of young people. According to the results, the most important carrier protein is α_2 macroglobulin, the second transferrin and the third albumin. FAA is hardly found at all in the α_1 globulin zone.

In the DPH users the total serum FAA was reduced while protein binding increased. A slight fall in protein binding is noted in the α_1 macroglobulin zone.

A review of the chromatogram analyses for the other patients (table III, Nos. 13-21) shows that, where the total serum FAA had been low (below 100 μ g/ml) the percentage of binding to proteins was high. The

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The Prevalence of Australia Antigen and Antibody in Haemophilia

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Abstract. 111 haemophilic individuals gave plasma to 4 tests for W. leishmania disease and 8 for the transformed particles (T. leishmania) to the cell to serum, 1 for antibody to serum in pregnancy) were examined by the immunoelectrophoretic (IOD) method for the presence of Australia antigen (Au(1)) and antibody among them. No Au(1) antibody or antibody was detected among the haemophilic. A 10-year-old child with a cell to serum had Au(1) antibody. Presumably a source for these serological results.

Key Words.
Australia antigen
and antibody
Electrophoretic
Haemophilia
W. leishmania
disease

The introduction of cryoprecipitation [14] substantially improved the management of classical haemophilia. The various modifications of the original procedure have made cryoprecipitation readily available in most centres. However, the discovery of Australia antigen (Au(1)) by Blumberg [2] has brought special attention to the problem [1, 9] of presence in plasma, cryoprecipitation and other plasma fractions [16, 22] and its association with disease and transmission of the virus [7, 8, 12] is indeed a serious threat to the management of haemophilia. It is essential to know if (1) it is detectable in the plasma, plasma and cryoprecipitation and (2) it is detectable in the plasma, plasma and cryoprecipitation [16] the extent of the virus does not seem to be a serious problem. Among the blood donors however, the prevalence of Au(1) is 6.0% [7]. It is a serious problem for a very serious problem.

From January 1971 when the prevalence of all blood donors for the Au(1) antigen and its antibody was determined from a community survey of 1,000 haemophilic subjects, a total of 1,000 blood donors and 1,000 blood donors were tested for Au(1) antigen and antibody. The results are as follows:

cidence of between 17 and 33% of anti-Au(1) antibody has been reported among haemophiliacs [19], we recently examined our patients for the prevalence of the Au(1) antigen and antibody among them. We also examined a matched group of other multiple transfused patients in order to establish the extent to which other factors in addition to the transfusion of probable Au(1)-containing blood affected our results.

Materials and Methods

Haemophilic patients There were 14 patients who had previously been diagnosed as either haemophilia A or B (factor VIII or factor IX deficient) and one case of von Willebrand's disease who had been attending the Haematology and other Clinics in the University College Hospital Ibadan [4-6]. Nine patients were originally examined and matched by age and socio-economic status with a control group (see below). Subsequently 6 other patients were included in the investigation but these were not matched with a control group.

The group comprised mild, moderate and severe haemophiliacs [10] aged between 2 and 22 years. Their mean requirement for transfusion of blood/blood fractions over a 3-year period was 22 U (range 5-60) red cells, fresh frozen plasma (FFP) and cryoprecipitate units inclusive. The various fractions used were usually derived from separate donors. Patients with high mean requirements were treated for surgical (including neurosurgical) complications of their disease state.

Other multiple transfused patients This group was made up of 8 patients aged between 1 and 25 years, 7 of whom at the time of our investigation had been attending the Paediatrics Department Anaemia Clinic and some had received multiple blood transfusions. The eighth patient attended the antenatal clinic for refractory anaemia in pregnancy. Their mean blood transfusion requirement was 5 U (range 1-14).

Serum samples During the preliminary tests, stored frozen plasma samples from the haemophiliacs were used. Subsequently freshly collected samples from both the haemophiliacs and the multiple transfused patients were used. All samples were left to clot at room temperature before they were centrifuged at 5000 rpm for 10 min and the serum samples separated. These were set up on the day of collection by the immuno-electro-osmosis method (IOD) of BRADARIC *et al* [1]. The remaining serum samples were frozen and preserved at -20 to -70 °C.

Results

Au(1) and anti Au(1) in haemophiliacs 14 haemophilic patients and one patient with von Willebrand's disease were tested for both the Au(1) and anti Au(1). None of the samples gave positive results for either the antigen or antibody.

Table 1. Summary of results of Austia's antigen antibody and other tests on haemophiliacs and other matched red-cell-transfused persons

| Haemophiliacs | | | | Other persons | | | |
|---------------|-----------|--------------------------|--|---------------|-----------|--|--|
| patient no. | age years | factor VIII or IX defect | transfusions received units ¹ | patient no. | age years | diagnosis | transfusions received units ¹ |
| 1 | 5½ | VIII | 6 | 1 | 6 | ITIS | 1 |
| 2 | 22 | VIII | 21 | 2 | 25 | refractory idiopathic thrombocytopenic purpura | 1 |
| 3 | 6 | VIII | 12 | 3 | 7 | ITIS | 7 |
| 4 | 3 | VIII | 83 | 4 | 2½ | ITIS | 1 |
| 5 | 10 | IX | 5 | 5 | 9½ | ITIS | 1 |
| 6 | 2 | VIII | 12 | | | | |
| 7 | 4 | VIII | 60 | 6 | 3 | ITIS | 2 |
| 8 | ½ | VIII | 5 | 7 | 1½ | ITIS | 2 |
| 9 | 10 | VIII | 14 | 8 | 8 | ITIS | 5 |

¹ Litres of blood transfused in haemophiliacs included whole blood, packed cells, cryoprecipitate and fresh frozen plasma. In others, only whole blood and packed cells were used.

Au(1) antigen and antibody in the control group of red-cell-transfused persons. In the control group one patient aged 2½ years who had received 100 ml of packed cells of Au(0) blood 5 months earlier, had an Au(1) (table 1).

Discussion

The prevalence of 6.0% of Au(1) antigen among our blood donors [2] is very high when compared with other published data for infants, 0.1%, among New York City volunteer donors [16]. It is therefore very likely that we had transfused some Au(1) positive blood¹ and transfused this some at least of our patients before we learned that on testing of blood donors for the Au(1) antigen and antibody to our hospital AIDS haemophiliacs and other persons whom we have examined, however, were negative both for Au(1) and the corresponding antibody even though 14 received a cumulative total of 22 U of red-cell-transfused plasma.

The results of low Au(1) antigen among our haemophilic patients are in agreement with those of others. Our results in respect of anti-Au(1) are, however, quite different. For instance, in environments with low Au(1) incidence both in the general population and among blood donors [16], anti-Au(1) has been reported in 29% of haemophiliacs (8 out of 28 patients), 28.6% (2 out of 7), 33% (7 out of 21), 13% (5 out of 39) and 17% (8 out of 48 patients) [7, 12, 18]. Our results cannot wholly be explained on the basis of our relatively small number of patients since the antigen is more than 60 times as frequent among our blood donors as among those in the populations where these other data were collected. We have used the same method by which other results were obtained. Moreover, it has been shown [17] that there is a 97-percent concordance between Au(1) results obtained by immunodiffusion and the more sensitive complement fixation methods.

Possible explanations for the higher prevalence of Au(1) among some healthy tropical population groups, lepromatous leprosy patients, leukaemic patients and children with Down's syndrome include the existence among such groups of widespread chronic anicteric hepatitis, genetic predisposition or defective immune mechanism with the result that they are more liable to infections [12]. We would include environmental factors in this list: for instance, exposure to mosquito bites [21]. None of our patients has evidence of any of the clinical states listed. But it is certain that at some time prior to this study, they had all been exposed to mosquito bites.

One possible explanation of our results could be that the total volume of blood and blood products which our patients have received is smaller than the volumes transfused into the haemophilic patients in other published series. It is difficult to comment on this point as relevant data are not available. It is, however, known that as little as 1 U of blood is adequate to infect the recipient and the antigen so acquired may persist for years. The results in our control series suggest that less than 1 U of blood can be infective. The 2½-year-old child with anti-Au(1) received 100 ml of Au(0) blood and 5 months later was found to carry anti-Au(1). The suggestion in this case is that she may not have acquired Au(1) to which she developed the antibody from the blood transfusion but probably from the ubiquitous insect ("mosquito") bite.

There is at present no evidence to suggest that our patients possess a more efficient immunological system which enables them to clear transfused Au(1) more readily than the apparently healthy blood donors, even

though it is known that adult Nigerians have higher levels of IgG and IgM than Europeans [13]. Further work in this area is receiving attention.

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Blood Group and Tissue Mosaicism in a Natal Indian Woman

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Abstract. A normal 45, XX karyotype was demonstrated in an Indian woman who has 2 appreciably equal populations of red cells and of leucocytes. She also has 2 distinct plasma types. She is a normal female and has borne 4 healthy children. The APO, G₆PD and IgG groups of her 2 red cell populations differed, but both were (a+b+) although she is a secretor of B and H₂ only once. The relationship between this and the 7 examples of diploerythrocytosis previously reported in the literature is discussed briefly.

Key Words:
Blood group mosaic
Diploerythrocytosis
Karyotype
Mosaicism
Tissue mosaic

The condition of diploerythrocytosis was first recognized in man in 1962 by GARTNER *et al.* [8] in an XX/XY hermaphrodite who had blood group mosaicism. Since then 6 further cases have been recorded, 2 of them with additional tissue mosaicism [1, 5, 11, 16]. Five were Caucasians [1, 5, 7, 10, 11, 14] and one was a Negro American Caucasian [1, 16]. Some evidence of hermaphroditism and XX/XY karyotypes was present in 6 of the cases [1, 4, 5, 7-11, 14, 16] and one a male conceptus was a mosaic for leucocytes [11]. Only the original case was enumerated as a female, the 2-year-old male of GARTNER *et al.* [8, 9]. Diploerythrocytosis is believed to be the result of separate fertilization of one or more ova by 2 sperm but the exact nature of the female contribution and when and how the products of fertilization co-develop into a single individual, is uncertain.

In this paper details are given of an eighth case of diploerythrocytosis recorded in 1968 [13] when an Indian woman was found to have blood group and tissue mosaicism. She was a normal female with an XX karyotype

and had borne four normal healthy children. A preliminary account of her blood groups was included in the review by RACE and SANGER [15].

Case History

When first seen the Indian woman, Mrs. T. R., who was a member of the Tamil sect of the Dravidian Indians, was 6 months pregnant with her fourth child. She was in good health and had come to the antenatal clinic for a routine examination. Mixed fields of agglutinated and unagglutinated red cells were seen in tests with anti-D. No history of a recent blood transfusion or bone marrow grafting was elicited and the ABO group of her fourth child at birth excluded the possibility of foeto-maternal transplacental haemorrhage (fig. 1). Dispermic mosaicism, as opposed to twin chimerism, was suspected when Mrs. T. R.'s mother stated that there had been no twin or unexplained issue at the birth of her daughter. Blood group tests on several occasions during the following 6 years, when Mrs. T. R. was not pregnant, confirmed the persistence of the 2 populations of red cells.

Results

Red cell studies In addition to the unexpected mixed agglutination with anti-D, further evidence of the presence of more than one type of red cell was obtained when Mrs. T. R.'s cells were grouped with anti-A, anti-C, anti-S and anti-Fy^b. In each test approximately 50% of the cells were agglutinated. Two distinct red cell populations were separated by using anti-A serum [2], and on a later occasion with incomplete anti-D serum followed by antiglobulin reagent. The groups of the separated cells were as follows: (1) A₁B, MNSs, P₁, T₁(a+), cde cde, Lu(a-b+), k₁ Kp(a-b+) Js(a-), Le(a+b-), Fy(a-b+), Jk(a+b-), Xg(a-), Do(a+), Co(a-b-), Vel+, I+. (2) B, MNs, P₁, T₁(a-), CDe cde, Lu(a-b+), k₁ Kp(a-b+) Js(a-), Le(a+b-), Fy(a+b-), Jk(a+b-) Xg(a+), Do(a+) Co(a+b-), Vel-, I+. Ashby counts [12] revealed 45% A₁B D-negative cells and 55% B D positive cells, supporting the original 50/50 estimate.

Lewis antigens and secretions Figure 1 shows that both the separated red cell populations were Le(a+b-). As Mrs. T. R. secreted B and H substances in her saliva, RACE and SANGER [15] pointed out that in these respects she is very similar to the Detroit example of dispermia [1, 16] who secreted A and H substances although all his red cells were Le(a+b-). BEATTIE *et al.* [1] deduced that the tissue formed from the zygote (100%) genetic product at fertilization in their case possessed the genes

sexe Lele and was capable of forming enough Le^s substance to coat the red cells of both populations. Since Mrs. T. R.'s father was dead and her mother was not tested for secretum it was impossible to decide whether the tissue producing her group B D-positive cells was genotypically Sexe Lele and her Lewis groups really Le(a-b+), as suggested by BEATTIE *et al.* [1] for the major (90%) A₁O component in the Detroit example, or was Sexe lele with cells Le(a-b-)

Two of Mrs. T. R.'s children were group O (fig. 1), strongly supporting the view that her reproductive cells and the tissue that was producing her group B, D-positive red cells were derived from the same original component at fertilization. If her A₁B, D negative red cell-producing tissue had been associated, she would have had only A or B children. It was unfortunately impossible to assess with certainty the maternal and paternal Lewis and secretor gene contribution in this case, but RACE and SANGER [15] suggested that Mrs. T. R.'s mother was very probably B Sexe, and her father had contributed A₁, se and O, Se sperm.

Leucocytes. HL-A grouping showed that there was an excess number of HL-A antigens present in Mrs. T. R.'s peripheral circulation. This is consistent with the existence of 2 different leucocyte populations. The antigens found were at the first (LA) locus, HL-A1, HL-A10 and W25 and at the second (four) locus, HL-A8, HL-A13 (doubtful) and W5

In all the reactions that were considered positive only half the number of leucocytes present reacted with each antiserum. This was in accordance with the 50:50 distribution estimated for the 2 populations of red cells. The Tunis example of dyspermy [4, 10], in which the lymphocytes were reported to differ by 2 HL-A haplotypes, was said to be genotype ac and bd in approximately equal proportions.

Skin examination. The skin of both of Mrs. T. R.'s thighs was completely covered with fine, intimately interwoven, striations of light and dark-brown pigmentation. The skin of her trunk was said to be marked in the same manner, but only the lighter of the 2 shades of pigmentation was observed on the head, arms and abdomen. This type of skin mottling is quite unlike the case reported by STRAZER *et al.* [1, 16] or that of COHEN *et al.* [5, 11] in which skin "patchiness" was described. The former (the Detroit example) had multiple small discrete areas of darker skin on the face, with fairly symmetrical welges and bands extending in a lateral direction from both the anterior and posterior midline on the trunk, buttocks and thighs. The latter (the Vancouver example) had large mottled areas, most conspicuous on the abdomen where they formed patches of

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Acquired Factor IX Deficiency

A Report of Two Cases

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Abstract. Coagulation studies in 2 patients revealed decreased factor IX activity. Family history of factor IX deficiency or bleeding disorder was not present and the past history of the patients did not disclose any evidence of congenital hemorrhagic disorder. They did not require any plasma or plasma product for the control of their bleeding diathesis and decreased factor IX activity returned to normal in a relatively short period of time. Factor IX deficiency was due to a circulating anticoagulant. With continued treatment, marked clinical improvement occurred in the second patient with some improvement of her coagulation studies, but it did not seem to affect the circulating anticoagulant level in the first patient.

Key Words:
Anticoagulants
Bleeding disorders
Blood coagulation
Factor IX deficiency

Acquired factor VIII deficiency has been reported in previously normal persons [1-9], in patients with collagen diseases, in patients treated with penicillin [1, 6-7], following electric shock [8] and in women following parturition [1-9]. Von Willebrand's syndrome [10-12] and factor IX deficiency [13-17] may also be acquired. We report here transient factor IX deficiency in a 14-year-old boy and in a 31-year-old female.

Case Reports

Case 1. A.C. (11.11.1953) - a 14-year-old male was referred to us by the pediatric service of the Hacettepe University Hospital (Children's Medical Center) when he developed pain in the ankles and some bleeding of 3 days duration. There was no family history of a bleeding tendency. The parents were concerned at a

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Acquired factor VIII deficiency has been reported in previously normal persons [1-5] in patients with collagen diseases, in patients treated with penicillin [1, 6-7] following electric shock [8] and in women following parturition [1, 9]. Von Willebrand's syndrome [10-12] and factor IX deficiency [13-17] may also be acquired. We report here transient factor IX deficiency in a 14 year-old boy and in a 31 year-old female.

Case Reports

Case 1 S. C. (HUH No 199336) a 14 year-old male was well until 10 days before being seen at the Hacettepe University Hospital Children's Medical Center, when he developed pain in the ankles and nose bleeding of 3 days duration. There was no family history of a bleeding tendency. The patient was circumcized at 4

years of age and had teeth extracted at 8 years of age without excessive bleeding. One brief episode of epistaxis occurred in early childhood; it did not require any treatment and did not recur. Physical examination showed a well-developed pale boy whose pulse was 108 per minute and blood pressure 120/80 mm Hg. A grade 2/6 systolic murmur was heard over the cardiac apex; the liver and spleen were not palpable and there were no ecchymoses or petechiae.

The hemoglobin (Hb) was 9.8 g/100 ml, hematocrit (Hct) 29%, WBC 10,300/mm³, with a differential count of 70% granulocytes, 1% bands, 2% monocytes and 27% lymphocytes and there was 1 normoblast per 100 WBC. Platelets were adequate on the peripheral smear and the red cells were normocytic, normochromic. The sedimentation rate was 45 mm/h (Westergren), ASO titer 500 Todd units, CRP, Coombs antiglobulin test and LE phenomenon were all negative. Total serum protein was 7.4 g/100 ml and electrophoresis showed albumin 41%, α_1 1.8%, α_2 19.4%, β 19.2% and γ globulin 18%. Immunoelectrophoresis was normal. Urinalysis showed 1+ proteinuria and microscopic hematuria. EKG disclosed slightly prolonged PR interval. X rays of the chest and extremities were normal and a throat culture was negative. Coagulation studies are reported separately.

The patient received corticosteroids, penicillin and iron. Three weeks later his Hb rose to 12.3 g/100 ml, Hct to 37% and his platelet count was 256,000/mm³. Intermittent nose bleeds occurred for the next 2 months. When last seen in April 1971, he was symptom free.

Case 2. M. A. (HUH No 208806), a 31-year-old housewife, was admitted to Hacettepe University Hospital on October 7, 1970, because of shortness of breath. She had been in good health until 18 months before admission when a cough and hoarseness developed during the early months of her first pregnancy, lasting 2 weeks; similar symptoms recurred 4 months later, again lasting 2 weeks. 13 months before admission she began having frequent nose bleeds and spontaneous ecchymoses, and 1 week before admission shortness of breath, cough, hoarseness and high fever occurred. On admission the physical examination showed BP 100/70 mm Hg, pulse 140 per minute, respiration 24 per minute and temperature 37°C. The skin and mucous membranes were pale and there were large ecchymoses over the buttocks. The throat was red and her voice was hoarse. The liver was palpable 8 cm below the right costal margin; the spleen was not palpable and the remainder of the examination was noncontributory.

The Hb was 8.7 g/100 ml, Hct 30%, WBC 9,000/mm³ with a differential of 3% myelocytes, 7% metamyelocytes, 5% bands, 20% polymorphonuclear (PMN) leukocytes, 8% monocytes and 57% lymphocytes. There were 18 normoblasts per 100 WBC. On the peripheral smear the red cells showed moderate anisocytosis and polychromasia and the platelets were decreased. Total serum protein was 6.5 g, albumin 3.0 g, globulin 3.5 g, bilirubin 0.8 mg, cholesterol 80 mg/100 ml, alkaline phosphatase 9.8 Bodansky units, SGOT 100 Karmen units and BSP retention was 22% after 45 min. Serum electrolytes, blood sugar, urea nitrogen and SGPT were normal. Serum iron was 199 μ g and total iron binding capacity 492 μ g/100 ml. Serum protein electrophoresis revealed albumin 34%, α_1 4%, α_2 24%, β 7% and γ globulin 31%. Serum antinuclear factor (ANF), LE cell phenomenon, cryoglobulin, cryofibrinogen and direct and indirect Coombs tests were negative. A bone marrow aspiration showed hypercellularity with erythroid hyperplasia and lymphocytosis. Direct

Table I Pertinent coagulation findings

| Tests ¹ | Case 1 | | | Case 2 | | | |
|--|---------|-----------------|---------|-----------|---------|----------|---------|
| | 8/25/70 | 8/27/70 | 9/7/70 | 11/2/70 | 11/4/70 | 11/27/70 | 6/1 |
| Clotting time, min (<12) | 45 | 4 | 75 | 4 | 7 | 5 | 9 |
| Bleeding time, min (<6) | 15 | ND ² | 2 | 5 | 17 | 20< | 16 |
| Retraction (2-4+) | 2+ | 2+ | 2+ | 0 | 0 | 0 | ND |
| Petechiometer (-) | - | ND | - | ND | ND | ND | ND |
| Quick prothrombin time, sec (<12) | 14 | 14 | 13 | 15 | 17 | 18 | 17 |
| PTT, sec (60-120) | 142 | 154 | 165 | 198 | 178 | 200 | 118 |
| Factor II, % (60-200) | ND | 79 | 113 | ND | ND | 67 | 60 |
| Factor V, % (60-200) | ND | 86 | 110 | ND | ND | 82 | 160 |
| Factor VII-X, % (60-200) | ND | 60 | 83 | ND | ND | 50 | 85 |
| Factor X, % (60-200) | ND | 62 | 109 | ND | ND | 59 | 119 |
| Fibrinogen, mg% (200-400) | ND | 397 | ND | ND | ND | ND | ND |
| Factor XIII (- to 1+) | ND | 1+ | 1+ | ND | ND | ND | ND |
| Platelets (150-400,000 mm ³) | ND | 278,000 | 265,000 | decreased | 203,000 | 66,000 | 132,000 |
| Platelet aggregation, sec (<18)ND | 11 | ND | ND | ND | ND | ND | ND |

¹ Numbers in parentheses indicate our normal values

² ND = not done

Table II TGT studies, sec¹

| | NS+
N ad pl ² | NS+
Pt ad pl | Pt S +
N ad pl | Pt S +
Pt ad pl |
|---------------|-----------------------------|-----------------|-------------------|--------------------|
| Case 1 | | | | |
| 8/25/70 | | | | |
| 8/27/70 | 93 | 92 | | |
| 9/9/70 | 89 | 82 | 131 | |
| 1/13/71 | 95 | 93 | 137 | 11 |
| | 91 | 92 | 139 | 116 |
| Case 2 | | | | |
| 11/2/70 | | | 95 | 114 |
| 11/4/70 | 10 | | | 92 |
| 11/27/70 | 85 | 98 | | |
| 6/1/71 | 87 | 84 | 22 | 242 |
| | 86 | 82 | 22 | 237 |
| | | 84 | 168 | 172 |
| | | | 92 | 84 |

¹ Only minimal substrate clotting times were recorded

² NS = normal serum, Pt S = patient serum, N ad pl = normal adsorbed plasma, Pt ad pl = patient adsorbed plasma

Table III PTT studies of case 1, sec

| | Before incubation | After 0.5 h incubation at 37°C |
|-------------------------------------|-------------------|--------------------------------|
| Normal plasma | 110 | |
| Patient's plasma | 165 | |
| ½ normal plasma + ½ VB ¹ | 114 | 113 |
| ½ normal plasma + ½ patient plasma | 114 | 162 |
| ½ patient plasma + ½ VB | 224 | 232 |

¹ VB = Veronal bufferTable IV TGT studies, sec Anticoagulant effect of the patient's serum before and after incubation for 0.5 h at 37°C¹

| | Before incubation | After 0.5 h incubation at 37°C |
|--|-------------------|--------------------------------|
| <i>Case 1 (Sept 9, 1970 serum)</i> | | |
| NS ² | 9.5 | |
| Pt S | 13.9 | |
| ½ NS + ½ VB | 10.9 | 10.6 |
| ½ NS + ½ Pt S | 10.9 | 17 |
| ½ Pt S + ½ VB | 17.2 | 32 |
| <i>Case 2 (June 1, 1971 serum)</i> | | |
| NS | 8.6 | |
| Pt S | 9.2 | |
| ½ Pt S + ½ Pt S (obtained Nov 27, 1970) ³ | 14.0 | 16.2 |
| ½ Pt S + ½ VB | 9.6 | 9.5 |

¹ Normal adsorbed plasma was used and only minimal substrate clotting times are recorded² Abbreviations as in tables II and III³ Kept frozen

levels in both 11% in the first patient (Sept 30, 1970), and 7.8% in the second patient (Nov 2, 1970 sample). Circulating anticoagulant(s) against factor IX was sought by mixing the patients sera and normal serum (table III, IV). The first patient's PTT findings suggested and the TGT results confirmed a circulating anticoagulant against factor IX, which disappeared by January 13, 1971. No anticoagulant could be demonstrated in the November 4, 1970 sample of the second patient's serum. However, when the sera obtained on November 27, 1970 and June 1, 1971 were mixed, the TGT became abnormal (table IV) suggesting the presence of an anticoagulant in the November 27, 1970 sample.

Comment

Acquired hemophilia A has been reported [1-5, 7, 8, 27-29] more often than acquired hemophilia B [1, 13-17]. Both of our patients are examples of the latter. The first patient, an adolescent boy with a negative family and past history of bleeding tendency, had a clinical picture consistent with rheumatic fever in which epistaxis is common. Because of the unusual severity of his nose bleedings further investigation was undertaken and the transient factor IX deficiency was found. In his case the factor IX deficiency was due to a circulating anticoagulant. In hereditary hemophilias, circulating anticoagulants occur most often following repeated transfusions [30, 31]. Patients with collagen diseases also may develop circulating anticoagulants against factor VIII [1, 10-12, 28]. However, we found in the literature only 2 cases of acquired factor IX deficiency due to circulating anticoagulants [14, 16]. It was not shown in 3 [13, 15, 32] and was most likely in one case of acquired factor IX deficiency [17]. There seems to be no good explanation for the sudden development of an anticoagulant against either factor VIII or IX but it is interesting that our patient had an illness consistent with rheumatic fever in which 'autoimmune' phenomena are known to occur [33].

Although von Willebrand's syndrome and rarely hemophilia A [34-37] and B [38, 39] do occur in females, in our second patient the factor IX deficiency was transient as is in all reported cases of acquired hemophilia B. There was only suggestive evidence of a circulating anticoagulant in one sample of her sera, all other samples showed the factor IX deficiency only. No satisfactory explanation for this was found. Apparently

in association with the factor IX deficiency, her clinical picture (characterized by fever, inflammation of the upper respiratory tract, hyperglobulinemia and perhaps, thrombocytopenia) was reminiscent of systemic lupus erythematosus a disease in which acquired hemophilia A [1-3], hemophilia B [17] and von Willebrand's syndrome [10] have been reported. ANF antibodies and LE phenomenon however, were absent. Acquired factor IX deficiency has been reported following viral infections, such as viral hepatitis [14] and although the clinical picture here was not suggestive of a viral infection, the possibility of a concomitant infection cannot be excluded without specific studies which we were not able to carry out. The role of pregnancy is also unclear, although acquired hemophilia A has been reported [1, 9] following parturition.

Thrombocytopenia and prolonged bleeding time may occur in myeloid metaplasia [40] which our patient had, but it would be difficult to explain the factor IX deficiency on this basis alone, because of the transient nature of the latter. Factor IX deficiency can occur in disseminated intravascular coagulation [41], but this too is unlikely in the case under discussion since there was no evidence of factor VIII or other clotting factor deficiencies when the factor IX level was low.

To date, all reported cases of acquired factor IX deficiency have recovered and this was true in our patients. The first patient with acquired factor IX deficiency [14] was treated with ACTH and the beneficial effect of corticosteroids was stressed in 2 other patients [15, 32]. Marked clinical improvement occurred in our second case with steroid therapy, but the value of this treatment in our first patient is very questionable since the factor IX deficiency disappeared several months after the steroids were discontinued. Clot promoting effect of corticosteroids is well known [42, 43] and their effect on factor IX level in a patient with Sheehan's syndrome has recently been reported [32]. But their efficacy in acquired factor VIII and IX deficiency has been questioned by others [1, 4, 7, 9, 28]. Acquired factor IX deficiency is a rare disorder. Perhaps this is more apparent than real, as the discovery of 2 cases in a short time may indicate. Investigation of 'minor' bleeding tendencies may lead to discovery of more cases.

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Haemoglobin D Punjab in a Cuban Family and its Interaction with Haemoglobin S

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Abstract A Cuban child carried 2 abnormal slow moving haemoglobins and suffered from severe haemolytic anaemia. Haematological studies of the family and chemical analysis of the abnormal haemoglobins indicated that he was a case of sickle-cell haemoglobin D disease, which is produced by the interaction between Hb S and Hb D Punjab. This is the first case of Hb D Punjab found in Cuba. The clinical picture of the patient is discussed.

Key Words

Haemoglobinopathies
Hb D Punjab in Cuba
Hb S
Sickle-cell Hb D disease

The classification haemoglobin D (Hb D) comprises the haemoglobin variants which are indistinguishable from Hb S in starch gel electrophoresis at pH 8.6 and in chromatography, but do not show the characteristic insolubility of Hb S in the reduced form, and thus do not have the ability to cause sickling [6]. A number of such haemoglobin variants structurally different have been described [4]. Among them Hb D Los Angeles, or Hb D Punjab is the most common since it is widely distributed in the Sikhs of Punjab [8]. Sporadic cases have also been found in European countries as well as in the United States, and recently one family has been found in Thailand [4, 13]. It has been suggested that this haemoglobin mutant has been exported from India, specially to countries which have had economic and political contacts with this country, although the possibility of independent mutations cannot be excluded.

The interaction between Hb D Punjab and Hb S is known as 'sickle-cell haemoglobin D disease', and in fact this Hb D was first described by ITANO [7] in a patient suffering from this disease. Many cases of sickle-cell haemoglobin D disease have been reported, although only in a

few of them Hb D was chemically identified. The main feature of this disease is that of an haemolytic anaemia resembling sickle cell anaemia in the mildest form, but the symptomatology of the cases so far reported presents a wide degree of heterogeneity. We want to report a case of Hb D Punjab found in a Cuban family and its interaction with Hb S.

Case History

DCF was admitted at the William Soler Hospital in Havana in January 1968 when he was 3 months old. He was first noted pale by his mother at the age of 1 month and diagnosis of anaemia was made in a Hospital of his locality. At the time of his admission he was remarkably pale, a slight jaundice was observed, the liver and the spleen were 6 and 11 cm below the right and left costal margins respectively. There was no history of anaemia in either parents. The father was a mestizo and the mother was a white descendant of immigrants from Spain.

Haemoglobin 4.5 g%, Leucocytes 10,921. Differential count: neutrophils 35%, basophils 2%, metamyelocytes 4%, myelocytes 2%, monocytes 5%, lymphocytes 52%. Peripheral blood showed marked hypochromia, anisocytosis, poikilocytosis, drepanocytes, polychromatophils, Howell Jolly bodies, target cells, basophilic stippling and normoblasts. Serum iron 174 μ g%, Bilirubin total 1.2 mg%, direct 0.155%. Bone marrow showed marked erythropoietic activity with megaloblastic changes and the iron staining was intensely positive. The electrophoretic pattern of his hemolyzate was that of an homozygote for Hb S with 12% foetal haemoglobin.

During his stay in the Hospital he had persistent thrombocytopenia and very low haemoglobin values, for which several transfusions were required and several months later splenectomy was decided after which a postoperative *Alebsiella aerea* infection developed. In the meantime the parents were studied (see Results and Discussion) and the previous diagnosis of sickle cell disease was changed to that of sickle cell haemoglobin D disease.

The propositus has been followed at the outpatient clinic. His physical growth was acceptable, but he showed slight mental retardation. After splenectomy he has had several infections in the upper respiratory tract, kidneys, skin, lungs and a severe bacterial meningoencephalitis. He was transfused during some of these infection episodes but he did not need any transfusion in between. The haemoglobin fluctuated between 5 and 7 g% and the reticulocyte count between 6 and 18% with many normoblasts in the peripheral blood. The liver remained 12 cm below the costal margin. Recently he was admitted to the Hospital for bone pains, but painful crises has been noticed seldom so far.

Methods

Routine haematological data were obtained by standard methods [3]. Starch gel electrophoresis was carried out at pH 8.6 using the discontinuous buffer of POULIK

Table 1 Haematological data of the family

| | Hb
g% | PCV
% | RBC
$\times 10^6/\text{mm}^3$ | MCV
μm^3 | MCH
μg | MCHC
% | Reticu-
locytes
% | Serum
iron
$\mu\text{g}\%$ | HbF
% | Solu-
bility
test |
|------------|----------|----------|----------------------------------|------------------------|----------------------|-----------|-------------------------|----------------------------------|----------|-------------------------|
| Father | 13.4 | 42 | 4.60 | 91 | 29 | 31.8 | 1.2 | 95 | 0.7 | positive |
| Mother | 12.5 | 40 | 4.30 | 90 | 29 | 31.2 | 1.8 | 111 | 0.5 | negative |
| Propositus | 6.9 | 24 | 2.05 | 120 | 34.5 | 28.7 | 12.1 | 174 | 12.2 | positive |

[9] The solubility test was done according to the method of IRANO as modified by GOLDBERG [5]. Foetal haemoglobin was quantitated by the alkali denaturation method of BETKE *et al* [2]. Chromatographic purification of the abnormal haemoglobin was obtained on diethylaminoethyl (DEAE) cellulose, using for the elution a linear ionic gradient of tris phosphate buffer at pH 8.5 [4]. Tryptic digestion of the heat-denatured purified abnormal haemoglobin was performed at 38 °C, and the peptide mixture obtained analyzed by the fingerprinting technique of INGRAM as modified by BAGLIONI [1]. The standard staining techniques for the identification of peptide spots and for specific amino acids were used. Peptides to be eluted for further analysis were localized by staining the peptide map in 0.02% ninhydrin solution in acetone, containing 5% of a pH 6.4 buffer (pyridine acetic acid water = 25:1:224), and heating at 60 °C for 20 min. The peptides were eluted with 6N HCl and hydrolyzed at 107 °C for 24 h *in vacuo*. After evaporation the sample was dissolved in water and the amino analysis performed in a Beckmann amino acid analyzer model 120B.

Results and Discussion

Clinical manifestations and haematological data of the propositus firstly suggested either a sickle-cell disease or a sickle-cell β -thalassaemia. Due to the high frequency of the S gene in our country the most likely diagnosis was that of sickle-cell anaemia, as indicated also by the electrophoretic pattern of the parents which was typical of the sickle-cell trait. However, the solubility test of the father was positive, whereas the mother's haemolyzate did not show any insoluble haemoglobin. It was thus concluded that the propositus was a carrier of both Hb S and another haemoglobin with the same electrophoretic mobility. The haematological data of the family are shown in table I.

Interactions between Hb S and other haemoglobins have been described each giving different manifestations. Thus, interactions between

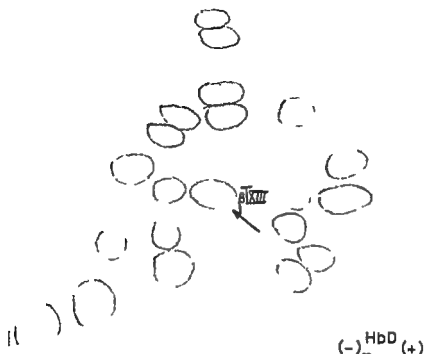


Fig 1 Fingerprint of the Hb D Punjab. The abnormal peptide is shown by arrow

Hb S and Hb C, Hb G, Hb D Ibadan have been found, the most serious from a clinical point of view being that between Hb S and Hb D Punjab. This entity is similar to sickle-cell disease, but shows a great degree of heterogeneity as far as clinical consequences are concerned. The clinical picture of the cases so far reported varies from an asymptomatic state to that of a severe anaemia [10-12]. Unfortunately, only in a few cases the Hb D was chemically identified.

In order to compare our case with the others previously reported, being sure that our haemoglobin was Hb D Punjab, the abnormal haemoglobin was purified from the mother's haemolyzate and analyzed as described under Methods. The fingerprint of this haemoglobin, shown in figure 1 is that of Hb D Punjab in which an aspartic acid is substituted for an asparagine in position 121 of the β -chain. This was confirmed by the amino acid analysis of the abnormal peptide. It was thus concluded that our propositus was a case of sickle-cell-Hb D disease. The patient presented an evolution particularly serious mainly during the first year of life. Levels of haemoglobin lower than 5 g% were maintained during

this period, which made it necessary frequent transfusions and kept the patient in a state of continuous danger

The other cases reported, although characterized by great variability, showed all the feature of painful crises which dominated the clinical picture. Our case is not characterized by such crises but he resembles to the patient reported by STURGEON *et al* [12], who also required splenectomy at 1 year of age. It is possible that the great splenomegaly was the cause that made worse the clinical picture and the anaemia during the first month of life. After splenectomy, although haemoglobin levels only raised to approximately 7 g%, the patient required blood transfusions only during severe infections which have been frequent, as described in the Case History. It is well known that this is not an unusual finding, for infections are common in haematological patients precociously splenectomized. We can say that our patient had the clinical course of a congenital serious haemolytic anaemia, which could have been easily confused with sickle cell anaemia in the most severe form and of precocious debut.

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The Value of Laparotomy and Splenectomy in the Staging of 56 Patients with Hodgkin's Disease

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Abstract The results of laparotomy and splenectomy in the staging of 56 patients with Hodgkin's disease are presented. Negative or dubious lymphangiograms were inaccurate in 37% of the patients who had abdominal lymph node involvement. Nonpalpable spleens were affected by Hodgkin's disease in 39% of the cases. Palpable hepatomegaly is not a good sign for predicting liver involvement, nor are BSP or alkaline phosphatase determinations. In 55% of the patients with involved spleen the liver was still free of disease. The prelaparotomy clinical stage had to be changed in 43% of the cases as a consequence of the postlaparotomy results, permitting a more accurate therapeutic approach.

Key Words
Hodgkin's disease
Laparotomy in
Hodgkin's disease
Splenectomy in
Hodgkin's disease
Staging in
Hodgkin's disease

Since EASSON and RUSSELL [2], KAPLAN [5, 6], and PETERS [10] published their early results on radiotherapy in Hodgkin's disease, the possibility of 'cure' has been suggested. KAPLAN's experiments [5, 6] clearly demonstrated the existence of a tumoricidal level in the radiotherapy of Hodgkin's disease; tumor doses of 4,000 rads could eradicate the disease in the treated region with less than a 4-percent relapse rate.

Nevertheless, radiotherapy of affected regions and prophylactic irradiation of areas suspected of occult involvement did not prevent early relapse on some patients who suffered an unknown or undiagnosed degree of disease involvement in the abdomen. Despite lymphangiography, the abdominal cavity was still the most difficult body region as far as evaluation of the extent of Hodgkin's disease was concerned. Laparotomy

seemed the logical procedure to be taken in order to achieve a correct staging. More recently, GLATSTEIN *et al* [3] published their first results about the value of laparotomy and splenectomy in the staging of Hodgkin's disease. Since then other reports [1, 4, 8, 9, 11, 12] have consistently confirmed their findings.

Patients and Methods

This report contains the results of 56 patients with confirmed Hodgkin's disease who underwent laparotomy and splenectomy for the staging. The study which lasted from January 1971 to June 1972 was a joint effort carried out by three hematological centers in Barcelona: Escuela de Hematología Farreras Valentí (31 patients) and Departamento de Hematología de la Ciudad Sanitaria 'Francisco Franco' (25 patients).

All patients, whose diagnosis of Hodgkin's disease was previously established by biopsy, were subjected to the following protocol: (1) meticulous case history and physical examination, (2) chest X ray and whole lung tomography, (3) routine blood tests, bone marrow aspiration, plasma protein electrophoresis, routine urinalysis, (4) liver functions tests - SGOT, SGPT, alkaline phosphatase (AP) and BSP retention, (5) bilateral lower extremity lymphangiogram, and (6) a number of patients were subjected to liver and spleen scans.

Furthermore, in selected patients, whose clinical signs and symptoms so warranted skeletal X ray studies, pycnograms, peritoneoscopy and/or liver needle biopsy were performed.

Patients with evidence of disseminated disease (stage IV) and with any medical contraindications to elective abdominal surgery were excluded from the study.

The modified classification recommended by the Nomenclature Committee at the Rye Conference was utilized. Table I shows the histological classification of the 56 cases. Note the predominance of nodular sclerosis in females and of mixed cellularity in males. The study included a majority of patients in stages II and III (49 of 56) with an equal distribution of A and B cases. The youngest patient was 15 and the oldest 64.

Clinical criteria for determining abdominal spread of Hodgkin's disease included: (1) 'abnormal' lymphangiogram, considered abnormal only when enlarged nodes containing filling defects were present, evidence of small filling defects in otherwise normal sized nodes was regarded as equivocal but clinically nondiagnostic, (2) palpable splenomegaly. A smooth palpable hepatomegaly was not clinically considered as demonstrating liver involvement nor were abnormal BSP or AP values.

In all cases the surgical procedure was performed by the same team in each center and consisted of the following steps: (1) splenectomy, including removal of the splenic hilar region, (2) biopsy of the suspect periaortic and/or iliac lymph nodes when the preoperative lymphangiogram proved positive or equivocal, (3) exposure and exploration of the left periaortic nodes from the 12th thoracic to the 2nd lumbar vertebra and sampling of this area, (4) exploration of the porta hepatis, (5) exploration of the entire visible and palpable surface of the liver and sampling of two

Table I Histological classification of the 56 cases

| | Male | Female | Total |
|-------------------------|------|--------|-------|
| Lymphocyte predominance | 3 | — | 3 |
| Nodular sclerosis | 5 | 14 | 19 |
| Mixed cellularity | 20 | 13 | 33 |
| Lymphocyte depletion | — | 1 | 1 |
| Total | 28 | 28 | 56 |

wedge biopsies, one from each lobe, when no gross pathological finding was detected and (6) bilateral oophorectomy in young women. Tantalum clips were left as references when nodes were taken out and as ovarian markers after oophorectomy.

Results

Several important correlations between prelaparotomy and postlaparotomy findings have been studied.

Correlation of lymphangiogram and abdominal nodes (table II) In about 37% of the cases with a negative or dubious lymphangiogram, laparotomy discovered abdominal lymph node involvement. Most of them were located above L2 in the left periaortic region not usually filled by lymphangiography. In contrast, in 28% (7 out of 25 cases) with a positive lymphangiogram, node involvement could not be confirmed by laparotomy.

Correlation of palpable splenomegaly and spleen involvement (table III) Of 13 cases with palpable splenomegaly, 3 had no spleen involvement. Thus, palpable splenomegaly is not always an indication of spleen involvement. On the other hand, nonpalpable spleens can be affected with Hodgkin's disease, as shown by this study, where 17 out of 43 (39%) were abnormal.

Correlation of hepatomegaly and liver involvement (table IV) In 15 out of 21 patients the liver was palpable but not involved, indicating that a palpable liver is not a reliable clinical sign of hepatic involvement. On the contrary, a nonpalpable liver was involved in a small percentage of the cases (7%).

Correlation of elevated BSP retention and/or AP and liver involvement (table V) The liver was uninvolved in 4 out of 11 cases with abnormal BSP retention and/or increased AP, while in 5 of 38 patients with normal results it was affected.

Table II Correlation of lymphangiogram and abdominal nodes

| Lymphangiogram | Total | Abdominal nodes | |
|----------------|-------|-----------------|----------|
| | | uninvolved | involved |
| Negative | 17 | 11 | 6 |
| Equivocal | 10 | 6 | 4 |
| Positive | 25 | 7 (28%) | 18 |

} (37%)

Table III Correlation of palpable splenomegaly and spleen involvement

| Spleen | Total | Spleen | |
|--------------|-------|------------|----------|
| | | uninvolved | involved |
| Palpable | 13 | 3 | 10 |
| Not palpable | 43 | 26 | 17 |

Table IV Correlation of hepatomegaly and liver involvement

| Liver | Total | Liver | |
|--------------|-------|------------|----------|
| | | uninvolved | involved |
| Palpable | 21 | 15 | 6 |
| Not palpable | 35 | 29 | 6 |

Table V Correlation of elevated BSP and/or AP and liver involvement

| BSP and/or AP | Total | Liver | |
|---------------|-------|------------|----------|
| | | uninvolved | involved |
| Abnormal | 11 | 4 | 7 |
| Normal | 38 | 33 | 5 |

Correlation of spleen scan and spleen involvement (table VI) There is a poor correlation between a normal spleen scan and spleen involvement, 9 out of 15 patients (60%) had a diseased spleen but a normal scan. The correlation improves when the spleen scan indicates pathological findings

Table VI Correlation of spleen scan and spleen involvement

| Spleen scan | Total | Spleen | |
|-------------|-------|------------|----------|
| | | uninvolved | involved |
| Abnormal | 4 | 1 | 3 |
| Normal | 15 | 6 | 9 |

Table VII Correlation of liver scan and liver involvement

| Liver scan | Total | Liver | |
|------------|-------|------------|----------|
| | | uninvolved | involved |
| Abnormal | 8 | 3 | 5 |
| Normal | 13 | 9 | 4 |

Table VIII Correlation of spleen involvement and liver involvement

| Spleen | Total | Liver | |
|------------|-------|------------|----------|
| | | uninvolved | involved |
| Involved | 27 | 15 | 12 |
| Uninvolved | 29 | 29 | 0 |

Table IX Reclassification of stage 24/56 (43%)

| | |
|-----------------|----|
| Stage advanced | 16 |
| Stage reduced | 8 |
| Stage unchanged | 32 |
| Total | 56 |

Correlation of liver scan and liver involvement (table VII) The liver scan is more reliable when it shows normal findings, 9 out of 13 normal liver scans had the liver uninvolved (70%). However, from 8 abnormal scans, the liver proved disease free in 5

Correlation of spleen involvement and liver involvement (table VIII)
In 12 out of the 27 cases with involved spleen, involvement of the liver was also present, but in no case where the spleen was normal, was the liver affected

Table IX summarizes the number of patients who required a postlaparotomy stage reclassification in 16 cases the stage was advanced, in 8 cases was reduced and in 32 cases remained unchanged Thus, 24 out of 56 patients (43%) needed restaging

Complications

One death occurred among the cases, a IIIB stage patient who died a month after laparotomy due to necrotizing enteritis One patient had to be relaparotomized one year after the original exploration because of intestinal obstruction and has had no further complications Two cases presented high fever a few days after laparotomy, one of them due to pneumonia A few patients presented wound infections of short duration It is surprising how well the great majority of patients with confirmed Hodgkin's disease tolerated and recovered from a major surgical procedure

Discussion

This report confirms previous publications on the value of laparotomy and splenectomy in the staging of Hodgkin's disease [1, 4, 8, 9, 11, 12] ROSENBERG [12] found that approximately 25% of spleens, unsuspected as involved clinically turned out to have Hodgkin's disease when studied carefully by the pathologist Conversely, approximately 50% of spleens judged to be clinically enlarged did not contain Hodgkin's disease when examined histopathologically Our results are somewhat different, showing a better correlation between palpable and affected spleens (10 spleens affected out of 13 palpable splenomegales), but a poorer one between nonpalpable and normal spleens (39% of nonpalpable spleens were affected) Possibly ROSENBERG's criteria of 'clinically enlarged spleen' included radiologically enlarged spleens The survey revealed that 4 cases with a spleen less than 200 g had spleen involvement In 2 cases there was only one pathological node visible macroscopically with no other evidence of disease in the abdominal cavity Another case had only one pathological node in the splenic hilus and no other abdominal finding

The correlation of involvement of the liver and spleen is similar to that of the Stanford group [12]. No examples of involvement of the liver without concomitant involvement of the spleen were found. On the contrary, of 27 cases with involved spleen, 15 cases (55%) had normal livers. In a previous study by GLATSTEIN *et al* [3] only 25% of cases with spleen involvement had a normal liver. Our results are more in keeping with the experience of AISENBERG *et al* [1] who emphasizes the important role of the spleen and splenic hilar nodes as being possibly the first intraabdominal regions to be affected when Hodgkin's disease begins at the supradiaphragmatic level.

BSP retention and AP determination are very poor in predicting the pathological involvement of the liver. Likewise, spleen and liver scans are not usually very helpful in predicting involvement of these organs. Only a normal liver scan can occasionally be useful. The above results are also in agreement with other publications [7, 13].

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Erythrodermia, Mycosis fungoides, Skin Reticulosis – Autonomous Disorders of the Monocytopoietic Macrophage System?¹

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Abstract Detailed studies of monocytopenesis and of blood monocytes were carried out in patients with erythrodermia, mycosis fungoides and skin reticulosis. A uniform pattern of several fundamental abnormalities was evaluated: (1) excessive monocytopenetic hyperproliferation focusing on promonocytes which exhibited lymphocyte size; (2) premature monocyte release from the bone marrow into the blood; (3) marked increase of pyknotic monocytes circulating; and (4) frequent occurrence of blood leukocytes which were characterized by large round nuclei, absence of NaF sensitive esterase and high DNA synthesis activity. There was some evidence indicating that these cells represent promonocytes with low degree of cytoplasmatic differentiation. This pattern of monocytopenetic abnormalities was uniformly present in virtually all patients. It did not correlate with the clinical diagnosis or with the extent of skin lesions.

Key Words

Disorders of
monocytopenesis
Erythrodermia
Kinetics of
monocytopenesis
Monocytes
Mycosis fungoides
Skin reticulosis

Erythrodermia, mycosis fungoides and skin reticulosis share several characteristics in common. The clinical course resembling that of malignant diseases, interchanges in symptomatology, and more or less widespread autonomous cellular skin infiltrations which are predominantly composed of histiocytes. It is well established today that histiocytes of normal skin, as well as macrophages appearing at the site of inflammatory reactions, arise from blood monocytes. The monocytes, in turn, are regenerated from promonocytes in the bone marrow [4, 6, 13, 16, 19,

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20] Therefore, in order to elucidate the nature of the above-mentioned diseases whose pathogenesis is not well defined, a detailed study of monocytopenia and blood monocytes was carried out

Materials and Methods

Subjects Consent for the investigations was obtained from 9 patients, mean age 59 years (range 39-70 years). The disease involved the whole integument in 7 patients, 3 cases with erythrodermia, 3 mycosis fungoides, and 1 reticulosis of the skin. In another patient with skin reticulosis skin affections were localized in the back and left upper arm. Still a further case of skin reticulosis was studied during partial remission following radiotherapy. Skin lesions were restricted to the right forehead and upper part of the nose. The patients examined had neither severe concomitant diseases nor signs of infection. Treatment with corticosteroids or cytotoxic drugs was discontinued at least 3 weeks prior to the study.

Examination of blood leukocytes Routine cell counts were performed using a Coulter Counter. 400 white cell differential counts were made on May-Grünwald Giemsa stained smears. Aliquots of 4 ml venous blood were mixed with Na_2EDTA plasmagel (1.107 g Na_2EDTA +14 g NaCl in 100 ml distilled water+100 ml plasmagel) and $10 \mu\text{Ci } ^3\text{H TDR}$ (specific activity 5 Ci/mm). The mixture was allowed to sediment for 30 min. Subsequently, the supernatant was pipetted off and spun at 160 g for 5 min. Concentrated leukocyte smears suited for microscopic evaluation were prepared from the sediment. These smears were submitted to May Grünwald staining, demonstration of naphthol AS-D-chloroacetate esterase [7], NaF resistant and NaF sensitive naphthol AS-D acetate esterase [14, 15].

500 polychrome stained monocytes were arbitrarily differentiated into three groups according to nuclear morphology: (1) monocytes with large round or oval nuclei, (2) with reniform or slightly folded (intermediate forms) and (3) distinctly folded nuclei.

Indices of reaction intensity for naphthol AS-D-chloroacetate esterase were established for each of the three types of blood monocytes by arbitrarily classifying 500 cells into four groups according to reaction activity: 0 = negative, 1 = slightly positive, 2 = medium positive and 3 = strongly positive. The activity index was calculated as follows: sum of the products of cell number within a group times group number related to 100 monocytes.

Smears exhibiting the combined reactions of NaF-sensitive and NaF-resistant naphthol AS-D acetate esterase were submitted to dipping film autoradiography (Ilford L4, 13 days exposure) and consecutive staining of nuclei with hemalaune (15 min) through the film layer. Hemalaune adsorbed by the film was carefully leached out by incubation in lukewarm water. In these preparations 2000 cells demonstrating positive enzyme reaction were scanned and divided with regard to nuclear morphology, enzyme reaction intensity (1 = slightly positive, 2 = medium positive, 3 = strongly positive) and $^3\text{H TDR}$ incorporation *in vitro* (nuclei overlaid by more than 4 grains were judged as being labeled). Enzyme activity indices were calculated as described above.

Examination of monocytopoiesis is reported in detail elsewhere [11] therefore only the principle of the study and important selected data are considered in this present paper. Bone marrow spicules washed free of contaminating blood were incubated in autologous serum, containing $12 \mu\text{Ci } ^3\text{H TDR}$, for 30 min at 37°C . Subsequently bone marrow smears were prepared. Some of the preparations were submitted to polychrome staining for establishing bone marrow differential count (1 000 nucleated cells were scanned). Some smears were used to demonstrate the monocytic series by use of combined reactions of NaF-sensitive and NaF resistant naphthol AS-D-acetate esterase [14, 15]. The relative number of promonocytes was determined by recording 3 000 nucleated bone marrow cells. Some smears, demonstrating this enzyme reaction, were submitted to autoradiographic processing using Ilford L₄ dipping emulsion and 13 days exposure. Following nucleus staining with hemalaune $^3\text{H TDR}$ labeling indices (LI) were determined by recording 1 000 promonocytes. Nuclei overlaid by more than 4 grains were judged as being labeled (mean background 1.1–2.3 grains per nucleus).

According to nuclear morphology promonocytes were divided somewhat arbitrarily into four groups: type I = lymphocyte like cells bearing small round or oval nuclei; type II = cells of myelocyte size with large round or oval nuclei; type III = cells of myelocyte size with large reniform or slightly folded nuclei, and type IV = cells of myelocyte size with distinctly folded nuclei.

The total marrow pool of hematopoietic cells and pools of different monocyte precursor types were calculated on the basis of data given by DONOHUE *et al* [2]. Monocyte birth rate (BR) by mitotic division within the intramedullary proliferation pool of monocytopoiesis was calculated according to the formula reported by CRONKITE *et al* [1]. $\text{BR} = \text{cell number within a compartment times LI divided by DNA synthesis time } (T_s = 10 \text{ h [11]})$

Results

The data evaluated in the patients exhibited a rather uniform pattern of abnormalities showing no specific correlation to the clinical diagnosis or to the extent of the skin lesions. Therefore, it seemed justified to treat the data statistically as samples of one population.

Blood monocytes Moderate and marked monocytosis, ranging from 585 to 1,275 monocytes/ μl , was present in 6 of 9 patients. Monocytosis was caused mainly by the increase of round or oval nucleated monocytes reaching 4 times the normal values (table I). The reaction of naphthol AS-D-chloroacetate esterase was intensified whereas the reaction of NaF sensitive naphthol AS-D acetate esterase was slightly depressed. $^3\text{H TDR}$ labeling indices of cells demonstrating positive reaction of NaF sensitive esterase were normal (table I). No atypical leukocytes were detected in May-Grunwald stained blood films or concentrated leukocyte smears.

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Table 1 Counts and functional characteristics of blood monocytes, mean, SD (in parentheses) of 9 patients (P) and 10 healthy individuals (N)

| Blood count/ μ l | Total | | Nucleus morphology | | | | | | | | pyknotic | |
|--|--------------|--------------|--------------------|-------------|--------------|-------------|--------------|-------------|------------|--------------|----------|---|
| | round | | intermediate | | folded | | | | | | | |
| | P | N | P | N | P | N | P | N | P | N | P | N |
| Activity index of naphthol-AS-D-chloroacetate esterase | 668
(278) | 370
(110) | 127
(65) | 30
(10) | 196
(112) | 120
(45) | 334
(160) | 220
(85) | 56
(37) | 0.6
(0.6) | | |
| Activity index of NaF-sensitive naphthol-AS-D-acetate esterase | 99
(24) | 42
(11) | 105
(34) | 128
(58) | 78
(14) | 53
(21) | 105
(37) | 26
(11) | - | - | | |
| 3 H-TDR labeling index, % ¹ | - | - | 150
(38) | 205
(10) | 216
(28) | 230
(8) | 199
(23) | 218
(5) | - | - | | |
| | 0.2 | 0.2 | - | - | - | - | - | - | - | - | | |
| ¹ Cells demonstrating positive reaction of NaF-sensitive naphthol-AS-D-acetate esterase | | | | | | | | | | | | |

Table II Blood count and ^3H TDR incorporation *in vitro* of leukocytes demonstrating large monocyte-like nuclei, however, negative reaction of NaF sensitive naphthol AS-D-acetate esterase, mean, SD (in parentheses)

| | Patients | | | Healthy individuals | | | | | | |
|-----------------------------------|----------|-------------|-------------|---------------------|-----------|---|---------|---------|--------------|---------|
| | n | total | round | intermediate | folded | n | total | round | intermediate | folded |
| % | 9 | 100 | 85.2 | 7.4 | 7.4 | 5 | 100 | 73.1 | 15.4 | 11.5 |
| Blood count/ μl | 9 | 351 | 29.9 | 2.6 | 2.6 | 5 | 2.6 | 1.9 | 0.4 | 0.3 |
| | | (5.2-127.5) | (4.7-108.3) | (0.2-9.3) | (0.2-9.8) | | (0-4.7) | (0-4.2) | (0-1.2) | (0-0.7) |
| ^3H TDR labeling index % | 9 | 25.2 | 27.9 | 12.1 | 3.4 | 8 | 20.1 | 23.6 | 8.8 | - |

Table III Naphthol AS D-chloroacetate esterase reaction intensity and ^3H -TDR incorporation *in vitro* of large blood leukocytes (mean cell diameter $\approx 20 \mu\text{m}$) bearing round or oval nuclei (analysis of 1,200 cells from 4 patients)

| | Degree of reaction intensity | | | |
|------------------------------------|------------------------------|-----|-----|------|
| | 0 | 1 | 2 | 3 |
| Cells, % | 6.3 | 1.4 | 7.2 | 85.1 |
| ^3H TDR labeling index, % | 92 | 33 | 8 | 2 |

Pyknotic blood monocytes In concentrated leukocyte smears a small number of cells were observed exhibiting intense and homogenous reaction of NaF-sensitive esterase and pyknotic nuclei. These generally appeared as one or two structureless chromatin droplets without connecting chromatin bridges. The mean count of these cells, which were considered as pyknotic monocytes, reached 5.6/ μl (table I).

To our knowledge this cell type has never been described before. Therefore, blood samples of 10 healthy individuals were examined. Pyknotic monocytes were observed, too, however mean counts reached only 0.6/ μl .

Large leukocytes with monocyte-like nuclei but with the absence of NaF-sensitive esterase reaction were observed in concentrated leukocyte smears of the patients as well as in the normal individuals. The counts differed remarkably, yielding mean values of 35 cells/ μl in patients and 3 cells/ μl in controls. The largest cell diameter averaged $21.6 \mu\text{m}$ (range 18.7–26.7 μm), mean smallest cell diameter averaged $19.2 \mu\text{m}$ (range 16.0–22.7 μm). The mean frequency distribution of round or oval nucleated cells, cells with reniform and folded nuclei, was 85.2/7.4/7.4% (table II). ^3H -TDR labeling indices of round nucleated forms reached remarkably high values, approximating 28%. In contrast, cells with reniform and folded nuclei yielded 12 and 3%, respectively.

In an attempt to further characterize these enigmatic large leukocytes naphthol AS D-chloroacetate esterase and ^3H -TDR incorporation was evaluated simultaneously in individual cells. Table III summarizes the data obtained by analyzing 1,200 large leukocytes with cell diameters of approximately $20 \mu\text{m}$ and large round or oval nuclei taken from 4 patients. 85% of these cells showed intense enzyme reaction, however, low DNA synthesis activity. The remaining cell fraction demonstrated a sharp increase of DNA synthesis activity paralleling a decrease of en-

Table IV Monocytopoiesis of the patients compared with results of healthy individuals (pool n=9, LI and BR n=7)

| Monocyte precursors | | Patients
X (range) | Normal
X (range) | X patients
X normal |
|---------------------|------|-----------------------|---------------------|------------------------|
| Type I | pool | 261 (101-637) | 30 (18-54) | 8.7 |
| | LI | 37 (25-54) | 7 (2-17) | 5.2 |
| | BR | 8.7 (3.8-19.5) | 0.15 (0.05-0.39) | 58.0 |
| Type II | pool | 272 (192-361) | 176.5 (143-219) | 1.5 |
| | LI | 88 (49-71) | 10 (8-14) | 6.0 |
| | BR | 15.5 (9.8-19.1) | 1.67 (1.3-2.3) | 9.3 |
| Type III | pool | 149 (61-324) | 279 (207-432) | 0.5 |
| | LI | 72 (48-90) | 10 (7-13) | 7.1 |
| | BR | 10.2 (3.6-20.2) | 2.98 (2.1-4.8) | 3.4 |
| Type IV | pool | 177 (73-286) | 79 (56-137) | 2.2 |
| | LI | 70 (51-81) | 25 (22-28) | 2.8 |
| | BR | 11.9 (6.0-18.8) | 2.04 (1.4-3.8) | 5.8 |
| Total | pool | 838 (612-1,346) | 584 (439-775) | 1.5 |
| | LI | 55 (44-71) | 12 (9-14) | 4.6 |
| | BR | 46.4 (32.0-59.1) | 6.84 (5.1-9.5) | 6.8 |

Pool = cells $\times 10^6$ /kg body weight, LI = ^3H -TDR labeling index *in vitro* (%), BR = cell birth rate (cells $\times 10^6$ /kg/h)

zyme activity. Extremely high ^3H -TDR labeling indices yielding 92% were observed in enzyme negative cells.

Bone marrow examinations The myeloid-erythroid ratio shifted slightly in favor of the erythroid series in all but one patient. Three cases demonstrated moderate eosinophilia. Judging by polychrome staining, there was a substantial increase of reticulum cells in all patients. The relative numbers of these cells averaged 5.9% and ranged between 3.8 and 9.8% (normal mean 0.4%, range 0.1-0.9%).

Pools of promonocytes The total promonocyte pool was regularly increased and exceeded the normal values by a mean factor of 1.5 (table IV). This increase was predominantly caused by an expansion of the type I precursor compartment (type I precursors = small promonocytes of lymphocyte size and round or oval nuclei).

^3H -TDR labeling indices of all promonocyte types were regularly elevated. The average of the pooled precursors yielded 4.6 times the normal level (table IV).

Monocyte birth rate Enlarged pools, as well as a rise in ^3H -TDR labeling indices of promonocytes, produced a striking increase in the monocyte birth rate yielding about 8 times the normal values (table IV). Excessively high birth rates, exceeding the norm by factors of approximately 60, were evaluated in the compartment of the small lymphocyte-like type I promonocytes.

Discussion

A rather uniform pattern of abnormalities concerning monocytopoiesis and blood monocytes was observed in all patients examined. This pattern did not specifically correlate to the clinical diagnosis, e.g. erythroderma, mycosis fungoides and skin reticulosis, or to the extent of the skin lesions. The monocyte birth rate by mitotic division, within the monocytopoietic proliferation pool, was strikingly increased. This increase was caused by two components: the expansion of the promonocyte pool and a rise in their DNA synthesis activity. These results indicate a reduction of the cell generation time. The time gained by the shortening of the cell cycle allows additional mitotic cycles to be performed by the promonocytes during the passage through the proliferation pool. It is of interest to note that maximum hyperproliferation was associated with the small lymphocyte-like type I promonocytes. Within the compartment of these precursors, the monocyte birth rate yielded approximately 60 times the normal values.

Previous studies revealed that proliferation capacity of monocytopoiesis is only partially utilized under normal conditions. The proliferation reserve was predominantly associated with the type I precursor compartment [9]. The rise in monocyte birth rate within this type I precursor compartment was observed equally in patients with infections and inflammation. The rate of increase, however, was limited to only 20 times the normal levels.

The pattern of abnormalities of blood monocytes found in the present study corresponded partially to the pattern observed in patients with infections [10, 12]. According to previous studies, it is suggested that monocytosis was due to the rise in monocyte influx rate [10, 12]. The increased cell influx, in turn, was correlated with premature monocyte release from the bone marrow into the blood. This line of reasoning was evidenced by the following findings: (1) increase of relative and absolute numbers of monocytes with round or oval nuclei, (2) elevated naph-

thol-AS-D-chloroacetate esterase activity in combination with depression of NaF-sensitive naphthol AS-D acetate esterase activity

Two unknown and undefined leukocyte forms were detected in the patients' blood. Further study revealed the presence of such cells in the blood of normal individuals. The ratio of cell counts normal patient averaged 1:10. One of these leukocyte forms was considered to be a pyknotic monocyte. This conclusion based on two arguments: (1) strongly positive reaction of NaF sensitive esterase being homogeneously distributed within cytoplasm, and (2) nuclei appearing as structureless chromatin droplets. These nuclei resembled those of the pyknotic neutrophils [3, 5, 17]. The rise of pyknotic monocytes in the blood may either indicate impaired cell clearance or increased intravascular monocyte death rate. The latter explanation is supported by the discrepancy between excessive monocytopoietic hyperproliferation on the one hand and only mild monocytosis on the other.

Blood counts of a further leukocyte form, whose nature is still somewhat enigmatic, yielded an average of $35/\mu\text{l}$ in patients and $3/\mu\text{l}$ in normal subjects. These cells exhibited the following characteristics: (1) mean cell diameter $20\ \mu\text{m}$, (2) large round or oval nuclei, (3) complete absence of NaF-sensitive naphthol AS-D-acetate esterase, (4) negative to intense reaction of naphthol-AS-D-chloroacetate esterase, and (5) high DNA synthesis activity, maximal values being present in naphthol-AS-D-chloroacetate esterase negative cells. These cells could not be ranked into the granulocytic or the lymphocytic series. In the first instance because of the absence of the 'shift to the left' in differential count and the absence of naphthol-AS-D-chloroacetate esterase reaction in a certain cell fraction. In the second instance on account of the large cell diameters. The occurrence of intermediate forms between the questionable leukocytes and normal blood monocytes gives rise to the assumption that the questionable cells represent immature forms of the monocytic series. It seems likely that the cells by virtue of an early stage of cytoplasmatic differentiation did not demonstrate NaF sensitive esterase activity.

Implications of the findings with respect to the pathogenesis of diseases examined. Erythrodermia, mycosis fungoides and skin reticulosis appear, from the clinical point of view, as strongly related entities. This concept is directly supported by the present studies which reveal a uniform pattern of fundamental abnormalities of the monocytopoietic system in virtually all patients examined. The fact that the abnormalities of

monocytopoiesis did not correlate with the extent of dermal lesions gives rise to an additional assumption, namely, that the skin affections merely represent more or less occasional symptoms of an autonomous disorder concerning the monocyte-macrophage system

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Synthese von Hamoglobin, RNS und Proteinen in der normalen Erythropoese^{1,2}

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Abstract Quantitative photometric investigations on erythroblasts show (1) the most pronounced formation of RNA and haemoglobin takes place in young erythroblasts (2) Besides the synthesis of haemoglobin in mature erythroblasts no longer capable of mitosis new formation of RNA continues (3) In the case of erythroblasts of the G₂ phase with nuclei of various sizes, a critical concentration of haemoglobin is achieved, but not exceeded (4) The stem cells are transformed during the G₁ phase predominantly into large, but occasionally into small erythroblasts as well (5) Also in normal erythropoiesis, a varying number of generation cycles takes place

Key Words Cytophotometry, Erythropoiesis, Haemoglobin formation, Nucleic acids, RNS synthesis

THORELL [1] publizierte 1947 die ersten quantitativen Befunde über Veränderungen des Nukleinsäure-, Protein- und Hamoglobingehalts in der Einzelzelle im Ablauf der Erythroblastenreifung. Er schlug damit für die Erythropoese erstmals eine Brücke zwischen Biochemie [12] und Zellmorphologie. In der Zwischenzeit konnten durch zytophotometrische [2, 3, 8] und autoradiographische [9, 16, 17, 22] Untersuchungen direkte Angaben über die Neubildungsraten einzelner Zellbausteine gewonnen werden. Ziel der vorliegenden Arbeit ist es, gleichzeitig das Ausmass der DNS- sowie der zytoplasmatischen RNS-, Protein- und Hamoglobunvermehrung in den verschiedenen normalen Erythroblasten-

¹ Herrn Prof. Dr. Dr. h.c. H. E. Bock zum 70. Geburtstag gewidmet

² Mit Unterstützung der Deutschen Forschungsgemeinschaft

generationen unter Berücksichtigung der interphasischen Kerngrössenzunahme der Erythroblasten [4] durch Bestimmung der Eigenabsorption der genannten Substanzen bei verschiedenen Wellenlängen im nahen UV-Licht zu erfassen [vergl. 5]

Methodik

Blut und Knochenmarksausstriche zweier Kranker mit normaler Erythropoese bzw. leichter pulmonal bedingter Polyglobulie (Tab. I) wurden auf Quarzobjektträgern ausgestrichen und nach Luftrocknung in wasserfreiem Glycerin eingedeckt. Die Einstellung im UV Licht erfolgte mit Hilfe einer Bildübertragung auf einen Fernsehschirm. UV Absorptionsmessungen erfolgten bei den Wellenlängen 265, 280, 313, 405 bzw. 416 nm mit dem Zeiss Zytophotometer UMSP 1. Zur Bestimmung des Hämoglobinmaximums wurden fortlaufende Absorptionsmessungen zwischen 380 und 440 nm durchgeführt. Da die Bestimmung des unspezifischen Lichtverlustes gemessen bei 313 nm infolge der niedrigen Absorption erheblichen methodischen Fehlern unterlag, entschlossen wir uns einen Mittelwert, bestimmt aus zahlreichen Einzelmessungen für jedes Präparat, den anschliessenden Computerberechnungen zugrunde zu legen.

Für die Berechnung der DNS bzw. RNS und Proteinmengen wurden die von uns bereits früher angegebenen Formeln [6] verwandt:

$$\begin{aligned} \text{DNS} &= \frac{E_{265} - E_{280}}{E_{265} + E_{280}} \cdot \frac{1}{2} \cdot \frac{1}{\text{Ext.}} \cdot \frac{1}{\text{Pfad}} \cdot \frac{1}{\text{Konz.}} \\ \text{RNS} &= \frac{E_{265} - E_{280}}{E_{265} + E_{280}} \cdot \frac{1}{2} \cdot \frac{1}{\text{Ext.}} \cdot \frac{1}{\text{Pfad}} \cdot \frac{1}{\text{Konz.}} \end{aligned}$$

bez.

ben sich unter gleichzeitiger Berücksichtigung der spezifischen Extinktionsfaktoren für Proteine unter Zugrundelegung der von CASPERSSON und SANTESSON [7] angegebenen Werte

Die Karyometrie erfolgte durch Abzeichnen der Zellsfläche vom Zentrum des Bildschirms und anschliessender Planimetrie. Vom Patient We. wurden 1500 Zellen von Di. 1000 Zellen untersucht.

Fehlerdiskussion Der Fehler setzt sich zusammen aus der Genauigkeit des eingesetzten Extinktionskoeffizienten, der Wertung der unspezifischen Lichtabsorption sowie aus dem jeweiligen Verhältnis der Extinktion 265 und 280 nm d.h. für die Proteinbestimmung von der gleichzeitig vorhandenen RNS-Konzentration und umgekehrt. Unter Zugrundelegung eines 10prozentigen methodischen Fehlers in der Bestimmung von E_{265}/E_{280} beträgt für die Berechnung der Proteinmengen bei $E_{265}/E_{280} = 1.2$ der maximale Fehler 32% bei 1.4 50% für die Berechnung der DNS bzw. RNS Mengen bei einem Verhältnis E_{265} zu E_{280} 1.2 maximal 20% bei 1.4 maximal 15%. Ein weiterer Fehler entsteht schliesslich dadurch, dass die den Kern überlappende Zytoplasmakappe der Messung nicht isoliert zugänglich ist. Da das überlappende Zytoplasmavolumen den Quotienten aus der über dem Kern areal gemessenen Hämoglobinabsorption und der zytoplasmatischen Hämoglobinabsorption nicht überschreiten kann, wurden unter Zugrundelegung dieses Wertes mit Hilfe eines weiteren Computerprogrammes alle zytoplasmatischen Daten noch

Tabelle I

| | Patient | |
|------------------------------------|-------------|------------------------|
| | Di ♀ | We ♂ |
| Alter, Jahre | 61 | 67 |
| Diagnose | Dickdarm Ca | M Boeck, Lungenfibrose |
| Hb, g% | 12,4 | 17,1 |
| Erythrozyten, Mill/mm ³ | 3,9 | 5,2 |
| HbE, pg | 32 | 33 |
| Erythrozytendurchmesser, μ m | 7,5 | 7,2 |
| Retikulozyten, ‰ | 12 | 2 |
| Leukozyten/mm ³ | 11 900 | 7 600 |
| Thrombozyten/mm ³ | 260 000 | 244 000 |
| Diff Blutbild, % | | |
| Stab | | |
| Seg | 7 | |
| Eos | 66 | 2,5 |
| Baso | 0 | 62,5 |
| Lympho | 0 | 3,5 |
| Mono | 22 | 0 |
| | 5 | 25 |
| Sternalpunktat | | 6,5 |
| Zellzahl/mm ³ | | |
| Rote/100 weisse Zellen | 84 000 | 140 000 |
| Erythrop Reif z. | 20,5/100 | 38/100 |
| Sideroblasten, % | 175 | 183 |
| Serumeisen, μ g% | 35 | - |
| | 79 | 68 |

ein zweites Mal berechnet. Da die Ergebnisse nicht wesentlich differieren, beziehen sich die im folgenden gemachten Angaben auf Werte, die die Kernüberlappung nicht berücksichtigen. Da die Zelldicke im Einzelfall nicht bekannt ist, können Angaben der Konzentration nur auf Menge pro μ m³ Zellfläche angegeben werden. Der Vergleich dieser Werte ist dennoch möglich, da die Zelldicken im Ausstrichpräparat des Knochenmarks untereinander keine wesentlichen Schwankungen aufweisen [4].

Um alle genannten Daten gleichzeitig an derselben Zelle gewinnen zu können, musste auch auf eine Vorbehandlung mit Ribonuklease zur exakten Bestimmung des Kern DNS Gehaltes verzichtet werden. Jeweils wurden deshalb Kern-DNS+RNS Gehalt gemeinsam bestimmt. Der Anteil der RNS dürfte nach SANDRITTER *et al* [6] bei andern Blutzellen 10% betragen. Da der DNS Gehalt des Zellkerns nur zur Zuordnung der Zelle in eine bestimmte Zyklusphase verwandt wurde, dürfte diesem Fehler keine wesentliche Bedeutung zukommen.

Ergebnisse

In den beiden untersuchten Sternmarkpunkaten wies die Mehrzahl aller Erythroblasten einen Kern DNS (+RNS) Gehalt um 7×10^{12} g auf. An Hand des unterschiedlichen Kern DNS-Gehaltes wurden die zytoplasmatischen Hamoglobin-RNS- und Proteinbefunde der G_0 - bzw. G_1 -Phase (max. $10,0 \times 10^{12}$ g) der frühen und mittleren S-Phase (bis 14×10^{12} g) sowie der späten S- und G_2 -Phase zugeordnet. Die Befunde wurden dann der Kerngrösse als einem möglichen Mass der Zellreifung gegenüber gestellt.

Hamoglobin: Grosse G_0 - bzw. G_1 -Phase Erythroblasten lassen häufig eine sichere Hamoglobinaufnahme noch vermissen. In den Erythroblasten aller Zyklusphasen steigt der Hamoglobingehalt pro Flächeneinheit (Abb 1) mit abnehmender Zellkerngrösse an. Die höchste Hamoglobinemenge pro Flächeneinheit wird in den kleinsten G_0 - bzw. G_1 -Phase Erythroblasten erreicht, während in G_2 -Phase-Erythroblasten unabhängig von der Kerngrösse eine bestimmte Hamoglobinkonzentration nicht überschritten wird (Abb 1 unten). Die Hamoglobinzunahme in grossen G_0 - bzw. G_1 -Phase-Erythroblasten über S-Phase zu G_2 -Phase-Erythroblasten wird noch deutlicher, wenn man den Gesamthamoglobingehalt betrachtet.

Gesamtzellproteine: Proteingehalt pro Flächeneinheit und Gesamtproteingehalt verhalten sich in den verschiedenen Kerngrössenklassen und Zyklusphasen ähnlich wie das Hamoglobin. Die besonders niedrige Gesamtproteinkonzentration in den jungen bzw. grossen Erythroblasten weist auf eine niedrige Trockengewichtskonzentration in den jungen Zellen hin.

Zytoplasmatische RNS: In grossen Zellen nimmt der zytoplasmatische RNS Gehalt von der G_1 - über die S-Phase zur G_2 -Phase deutlich zu (Abb 2). Dies kommt insbesondere dann zur Darstellung, wenn man den RNS-Gehalt nicht der Kerngrösse sondern dem Kern-DNS-Gehalt in verschiedenen Erythroblastengrössenklassen gegenüberstellt. Die RNS-Neubildung nimmt während der Interphase mit zunehmender Ausreifung (d.h. kleinerem Zellkern) immer mehr ab. Als Folge der Halbierung des RNS-Gehaltes nach jeder Zellteilung besteht eine signifikante ($p < 0,05$) Korrelation zwischen zytoplasmatischem RNS-Gehalt und abnehmender Kerngrösse.

Der Quotient RNS zur Hamoglobinkonzentration sinkt mit abnehmender Erythroblastengrösse im Ablauf des Zellzyklus von G_1 über S

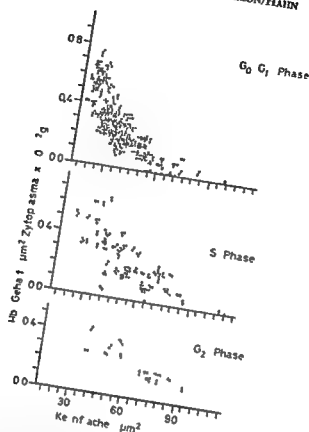


Abb 1 Hamoglobingehalt/ μm^2 bei verschieden grossen Erythroblasten aufgrund des Kern DNS (+RNS) Gehaltes in G_0/G_1 , S G_2 Phase Zellen getrennt Patientin D, 61 Jahre normale Erythropoese

nach G_2 . Das bedeutet dass in den jungsten d.h. grössten Erythroblasten eine starke interphasische RNS Neubildung und Einstrom ins Zytoplasma stattfindet während mit zunehmender Zellreifung (kleinerer Erythroblastenkern) die Hamoglobinsynthese immer mehr in den Vordergrund tritt. In den reifen Erythroblasten mit pyknotischem Zellkern kommt es aber neben der Hamoglobinsynthese noch zu einer Zunahme des zytoplasmatischen RNS Gehaltes. Das zeigt eine positive Korrelation ($p < 0.05$) von Hamoglobin und RNS Gehalt in der herausgegriffenen Gruppe mit niedrigem Kern DNS und RNS Gehalt (6.3 bis 6.7×10^{12} g). Bei dem Vergleich der Befunde an den beiden Sternalpunktaten zeigt sich dass der höchste Hamoglobingehalt pro Flächeneinheit in den

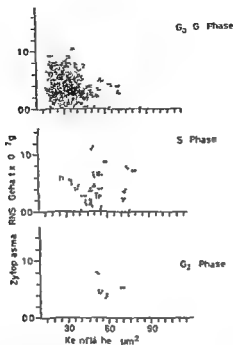


Abb 2 Zytoplasma RNS-Gehalt in verschiedenen grossen Erythroblasten aufgrund des Kern DNS(+RNS)-Gehaltes in G₀/G₁ S- G₂ Phase Zellen getrennt. Patientin D₁

kleinsten Erythroblasten bei D₁ höher als bei dem Patienten We mit pulmonaler Polyglobulie ist. Umgekehrt ist bei gleichem Verhalten im Ablauf der Interphase und mit zunehmender Zellreifung der absolute RNS-Gehalt bei We grösser als bei D₁.

Umschriebene Zellhaufungen bei unterschiedlichen Substanzkonzentrationen als Hinweiszeichen für verschiedene Erythroblastengenerationen konnten wir nicht nachweisen. Ursache hierfür dürften die fließenden Übergänge zwischen den für die vergleichenden Untersuchungen willkürlich abgegrenzten 3 Phasen des Generationszyklus entsprechend der kontinuierlichen DNS-Zunahme von G₀ bzw. G₁ nach S und G₂ sein.

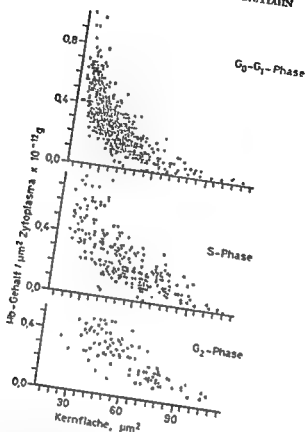


Abb. 1. Hämoglobingehalt/ μm^2 bei verschieden grossen Erythroblasten, aufgrund des Kern-DNS (+RNS)-Gehaltes in G_0/G_1 - S- G_2 -Phase-Zellen getrennt, Patientin Di, 61 Jahre, normale Erythropoese.

nach G_2 . Das bedeutet, dass in den jungsten, d. h. grössten Erythroblasten eine starke interphasische RNS-Neubildung und Einstrom ins Zytoplasma stattfindet, während mit zunehmender Zellreifung (kleinerer Erythroblastenkern) die Hämoglobinsynthese immer mehr in den Vordergrund tritt. In den reifen Erythroblasten mit pyknotischem Zellkern kommt ■ aber neben der Hämoglobinsynthese noch zu einer Zunahme des zytoplasmatischen RNS-Gehaltes. Das zeigt eine positive Korrelation ($p < 0,05$) von Hämoglobin und RNS-Gehalt in der herausgegriffenen Gruppe mit niedrigem Kern-DNS- und RNS-Gehalt ($6,3$ bis $6,7 \times 10^{-12}$ g). Bei dem Vergleich der Befunde an den beiden Sternalpunktaten zeigt sich, dass der höchste Hämoglobingehalt pro Flächeneinheit in den

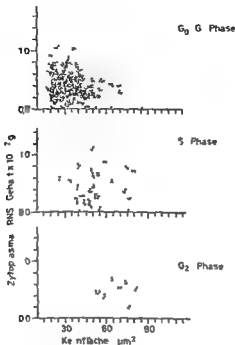


Abb 2 Zytoplasma RNS Gehalt in verschiedenen grossen Erythroblasten aufgrund des Kern DNS(+RNS) Gehaltes in G₀/G₁; S- G₁ Phase Zellen getrennt Patientin D₁

kleinsten Erythroblasten bei D₁ hoher als bei dem Patienten We mit pulmonaler Polyglobulie ist Umgekehrt ist bei gleichem Verhalten im Ablauf der Interphase und mit zunehmender Zellreifung der absolute RNS Gehalt bei We grosser als bei D₁

Umschriebene Zellhaufungen bei unterschiedlichen Substanzkonzentrationen als Hinweiszeichen für verschiedene Erythroblastengenerationen konnten wir nicht nachweisen Ursache hierfür dürften die fließenden Übergänge zwischen den für die vergleichenden Untersuchungen willkürlich abgegrenzten 3 Phasen des Generationszyklus entsprechend der kontinuierlichen DNS-Zunahme von G₀ bzw G₁ nach S und G₂ sein

Discussion

Zellen mit kaum nachweisbarer Hamoglobinsorption befinden sich aufgrund des Kern-DNS Gehaltes in G_0 - bzw. G_1 -Phase. Der völlige Mangel an Hamoglobin in diesen Zellen lässt sich am besten dadurch erklären, dass die durch Erythropoietin induzierte Umwandlung differenzierter Stammzellen [11, 13] in morphologisch erfassbare rote Vorstufen in der G_1 -Phase erfolgt. Da nur wenige grosse Erythroblasten in der G_1 -Phase vorliegen, muss man annehmen, dass mit der Umwandlung zu roten Vorstufen die Zellen bereit zum Eintritt in den Zyklus sind.

Neben grossen G_1 -Erythroblasten ohne nachweisbare Hamoglobinemengen finden sich auch einzelne kleinere Erythroblasten bis nahezu $55 \mu m^2$ (D1) bzw. $30 \mu m^2$ (We) Kernfläche, in denen noch nicht eindeutig Hamoglobin erfasst werden kann. Da bereits im ersten Zyklus der Erythroblasten Hamoglobin gebildet wird, muss man schliessen, dass Stammzellen eine Umwandlung nicht nur in grosse, sondern vereinzelt auch in kleinere Erythroblasten erfahren.

Zusammen mit der Umwandlung differenzierter Stammzellen in rote Vorstufen kommt es zu einer ausgeprägten zytoplasmatischen RNS-Zunahme besonders in den jungen, d.h. grossen Erythroblasten. Die Bildung DNS-abhängiger RNS [18] erfolgt unter Erythropoietinwirkung [10], es werden stark markierte r- und t-RNS verschiedener Sedimentationskonstanten nachweisbar [15]. Damit wird in der jüngsten Erythroblastengeneration alle RNS gebildet, die zur Produktion der neuen Zellproteine [14, 22] erforderlich ist und über die Aktivierung der Fermente der Häm synthese zur gesteigerten Hamoglobinsynthese führt [19]. Die RNS-Neubildung erfolgt nicht nur in allen teilungsfähigen Erythroblastengenerationen, wie das auch in autoradiographischen Studien mit 3H -Cytidin [9] nachgewiesen wurde, eine RNS-Neubildung erfolgt vielmehr zusammen mit der weiteren Hamoglobinsynthese auch noch in den reifsten nicht mehr teilungsfähigen Erythroblasten.

Die interphasische Hamoglobinzunahme scheint in den mittelgrossen Erythroblasten starker zu sein als in Proerythroblasten. Diese Befunde entsprechen den biochemischen Untersuchungen an den Dichtegradienten-getrennten Fraktionen des Knochenmarks [20].

Die Proteinsynthese erfolgt in allen Erythroblastengenerationen, Befunde die sich mit den biochemischen Untersuchungen decken [21]. Mit zunehmender Zellreifung nimmt dabei die Hamoglobineubildung einen grosseren Anteil an dem Gesamtproteingehalt an [12].

In der Gruppe der G_2 -Phase Erythroblasten wird eine kritische Hamoglobinkonzentration nicht überschritten. Man muss daraus schließen, dass die Zelle keinen weiteren Zyklus mehr durchläuft, sondern bei weiterer Hamoglobine Neubildung nur noch ausreift. Diese «kritische Hamoglobinkonzentration» wurde von STOHLMAN⁴ et al [16] als ein wichtiger Faktor in der Regulation der Erythropoese im Sinne eines Repressormechanismus auf die weitere DNS-Neubildung gedeutet. Die Tatsache, dass eine kritische Hamoglobinkonzentration, die nicht überschritten wird, in G_2 -Phase Erythroblasten unterschiedlicher Kerngrösse anzutreffen ist, zeigt, dass sich auch in der normalen Erythropoese nicht alle Erythroblasten gleichartig verhalten und eine konstante Zahl an Reifungsstufen bis zur endgültigen Ausreifung und Kernausstossung [17] nicht durchlaufen wird.

Alle an den Erythroblasten verschiedener Grössen und Zyklusphasen des Patienten We gewonnenen Ergebnisse wurden in einer vorläufigen Mitteilung [23] unter Berücksichtigung der interphasischen Grossenzunahme in einem Modell der normalen Erythropoese eingegliedert. Ausser des insgesamt höheren RNS-Gehaltes bei dem Kranken mit pulmonalbedingter Polyglobulie entsprechen die Befunde der beiden bisher untersuchten Kranken einander.

Zusammenfassung

Quantitative photometrische Untersuchungen an Erythroblasten zeigen: 1. Die ausgeprägteste RNS- und Hamoglobine Neubildung findet in jungen Erythroblasten statt. 2. Neben der Hamoglobinsynthese geht in den reifen und nicht mehr teilungsfähigen Erythroblasten auch eine RNS-Neubildung weiter. 3. Eine kritische Hamoglobinkonzentration wird von G_2 -Phase-Erythroblasten verschiedener Kerngrösse erreicht, aber nicht überschritten. 4. Die Stammzellumwandlung erfolgt in der G_1 -Phase überwiegend in grosse, vereinzelt auch kleine Erythroblasten. 5. Auch in der normalen Erythropoese werden danach bis zur Erythroblastenreifung eine unterschiedliche Zahl an Generationszyklen durchlaufen.

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Chromosome Studies in Paroxysmal Nocturnal Haemoglobinuria¹

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Abstract Chromosome studies have been performed in bone marrow and in venous blood cells of two patients with paroxysmal nocturnal haemoglobinuria (PNH). The frequency of aneuploidy in marrow cells was of 40 and 35%, respectively. Blood lymphocytes showed aneuploidy in only one patient. The data are consistent with the hypothesis that PNH can be a myeloproliferative disease, but no direct evidence has been found neither for a clonal composition of PNH nor for participation of all marrow cell lines.

Key Words
Chromosome anomalies
Karyotype
Myeloproliferative disorders
Paroxysmal nocturnal haemoglobinuria

Paroxysmal nocturnal haemoglobinuria (PNH) has been recently suggested as a 'candidate' myeloproliferative disorder [1]. Cytogenetic studies are relevant to this hypothesis in order to clarify if the metabolic defect(s) of red cells is (are) associated with one or more abnormal cell clones. Moreover cytogenetic findings can provide supplementary data to the open question if PNH is a disorder limited to erythropoiesis or if it also affects the other marrow cell lines [2, 3]. Previous chromosome investigations in PNH are quite rare [4-6], and more information is needed. The present paper reports cytogenetic findings in marrow and blood cells of two patients with PNH.

Patients and Methods

Patient 1 (DM) is a 24 year old woman, has been studied 3 years after diagnosis. All through this period she has been regularly followed up at our out patient clinic and she has been admitted at our hospital approximately every 2 months for need of blood transfusion. Patient 2 (MF), a 38 year old man, has been PNH

¹ Presented in part at the 23rd Congress of the Italian Society of Haematology

Table I

| | Patient 1 | Patient 2 |
|--|-----------|-----------|
| Haematocrit, % | 24 | 25 |
| Hb, g/100 ml | 6.15 | 6.75 |
| RBC/ μ l | 1,940,000 | 2,800,000 |
| Reticulocytes/ μ l | 184,300 | 181,000 |
| Neutrophilic polymorphonuclears/ μ l | 800 | 2,000 |
| Platelets/ μ l | 105,000 | 168,000 |
| Acidified serum test, % lysis | 48 | 41 |
| Acetylcholinesterase assay, Δ pH/h (normal range 0.5-1.0) | 0.27 | 0.28 |

bearer for 8 years. His need for blood transfusion was not as frequent as for patient 1.

The acidified serum test (Ham's test) and the acetylcholinesterase assay have been performed according to Dacie and Lewis [7]. Marrow cell metaphases have been obtained by a modification of T'no and Whang's method [8]. Venous blood cell metaphases have been obtained by a modification of the method of Moorhead *et al* [9].

Results

Pertinent laboratory data at the time of study are reported in table I. Figure 1 shows chromosome analysis of marrow cells of patient 1. Seven out of 20 metaphases have an abnormal number of chromosomes. Another metaphase is pseudodiploid, lacking one chromosome from group E₁₇₋₁₈, and having an extrachromosome in group D. Most of the remaining cells are hypodiploid, with chromosome loss from group E₁₇₋₁₈ (3 cells), group C (3 cells), group A₂, and D (2 cells each), group F, and G (1 cell each). Figure 1 also shows karyotype analysis from venous blood cells of the same patient. Again 13 out of 20 metaphases have 46 chromosomes, but one of them is pseudodiploid, lacking one chromosome from group G, and having an extra D. The remaining 7 aneuploid metaphases are spread on a basis going from 41 to 47 chromosomes. Here again as in marrow cells, there is some prevalence of 45 chromosome metaphases. Five cells lack one or more chromosomes from group C.

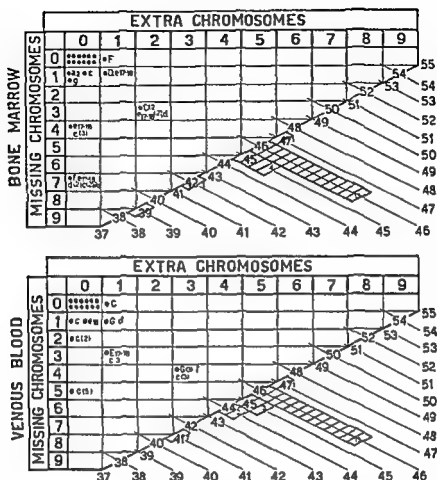


Fig 1 Patient 1 Karyotype analysis of marrow and blood cells. Number of extrachromosomes per metaphase in abscissa. Number of missing chromosomes per metaphase in ordinate. Each metaphase is represented by a black dot whose position in the graph depends on the number of extra and missing chromosomes. The metaphases with the same number of chromosomes are disposed along the same diagonal. Extrachromosomes are defined by capital letters, and missing chromosomes by small letters, the letters correspond to the chromosome group affected. On the right side of the graph the metaphase distribution according to chromosome number is shown by a histogram.

In patient 2 (fig 2), 13 out of 20 metaphases show a normal karyotype. One cell is pseudodiploid having two extra-chromosomes in group C, and one in group G, and lacking three chromosomes from group E₁₇₋₁₈. Two metaphases have one extrachromosome in group C and

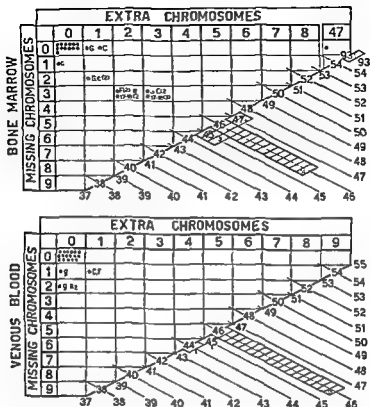


Fig 2 Patient 2 Karyotype analysis of marrow and blood cells. Interpretation as in figure 1

G, respectively. Chromosome loss affects only groups C and E₁₇₋₁₈. A cell with 93 chromosomes is shown at the top right of the figure. As to venous blood cell karyotype, there is a normal distribution around the mode, with one pseudodiploid cell, and two hypodiploid (44 and 45 chromosomes).

Discussion

In the two cases studied the frequency of aneuploidy is remarkably high 40% in patient 1, and 35% in patient 2. These findings partially

agree with previous reports [4-6]. Pooling together the data from the two patients, 14 marrow cells out of a total of 40 examined, are aneuploid or pseudodiploid. Chromosome loss affects mostly group E₁₇₋₁₈ (6 cells) and group C (5 cells), but it has to be noted that contemporary loss of E₁₇₋₁₈ and of C chromosomes appears only in two cells. On the contrary, most of extrachromosomes belong to groups C and G. There are no E₁₇₋₁₈ extrachromosomes. Venous blood cell karyotype of patient 2 can be considered to be normal, while in patient 1 35% of scored metaphases are aneuploid (one of them being pseudodiploid). In this case, loss of C chromosomes is striking (5 cells), but there are no cells lacking E₁₇₋₁₈ chromosomes as in marrow.

Marrow cell karyotype has been found to be normal in most haemolytic anaemias [4, 10], while it is frequently abnormal in myeloproliferative disorders [11]. Moreover, a significant aneuploidy has been found in refractory sideroblastic anaemia, another possible myeloproliferative disorder marked by a prominent defect of erythropoiesis [13 unpublished observations]. Therefore, the present findings are consistent with the hypothesis that PNH is a candidate myeloproliferative disease [1, 3]. Nevertheless, chromosome analyses have not provided any direct evidence for the presence of one or more abnormal cell clones. It is obvious that cytogenetic observations do not rule out the possibility that PNH is a clonal disease. PNH cells could have a normal or apparently normal karyotype and aneuploid or pseudodiploid cells could represent short living cell lines arising from PNH cells.

The ratio erythroid metaphases/granulocytic metaphases, determined on direct smears prepared from the same marrow aspirates and stained with May Grunwald Giemsa, by counting at least 100 consecutive erythroid metaphases, was 15/1 in patient 1, and 6/1 in patient 2. It is questionable to attempt any relationship between cytological distribution of mitoses in direct smears and chromosome preparations [12-15]. Nevertheless, this observation provides indirect evidence that some of the erythroid precursors have to be euploid, and some have to be aneuploid, while granulocytic precursors could be all euploid or all aneuploid, or both.

In conclusion, this study, while suggesting PNH as a myeloproliferative disorder, was unable to give direct evidence, neither for a clonal origin of PNH nor for participation of all marrow cell lines. Keeping in mind that PNH is an acquired disease, one could speculate if a hypothetical PNH inducing factor produces chromosome abnormalities as

well as metabolic defect(s), or if a cell population characterized by an abnormal genetic (chromosome) fragility is more successful in developing PNH. Whatever the truth, a pronounced genetic (chromosome) instability can explain the development and the establishment of abnormal clones [16], and then the development of acute leukemia [17-19].

The finding of a significant aneuploidy in blood lymphocytes of patient 1, who had no myeloid precursors circulating in blood, was rather surprising, also in view of previous reports [6]. This observation can suggest the possibility that chromosome instability in PNH, whatever the mechanism, is a pattern of non myeloid cells too.

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A New Unstable Haemoglobin: Hb Buenos Aires, β 85 (F1) Phe \rightarrow Ser

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MRC Abnormal Haemoglobin Unit, Department of Biochemistry, Cambridge

Abstract A new unstable haemoglobin, Hb Buenos Aires β 85 (F1) phenylalanine \rightarrow serine has been found in an Argentinian family. Four members were found to be heterozygous for this haemoglobin, and all suffered from a compensated haemolytic anaemia. This haemoglobin is the fifth example of an unstable variant resulting from the substitution of one of the phenylalanine residues in Hb A and underlines the importance of this large hydrophobic amino acid in maintaining the internal structure and stability of the molecule. The clinical and haematological findings of the effect on carriers are discussed in terms of the nature of the mutation, and the genetic features of phenylalanine substitutions in haemoglobin are examined.

Key Words

Haemoglobinopathies
Hb Buenos Aires
Unstable haemoglobins

A 17 year-old adolescent had been under observation for 5 years because of frequent attacks of jaundice associated with dark urine. The attacks were more severe when he was suffering from infections. As this history suggested an unstable haemoglobin as the likely cause, he was investigated for this possibility.

Methods

Haematological indices were measured using routine techniques [1]. Heat instability tests were carried out by diluting a haemolysate with 0.01 M tris-HCl buffer pH 6.8 and heating it for 60 min at 50 °C. Haemoglobin electrophoresis was carried out on starch gel at pH 8.6. The abnormal haemoglobin was isolated from stroma-free haemolysates by precipitation with isopropanol solutions as described by CARRELL and KAY [2]. The α and β -chains were separated according to the method of

CLEGG *et al* [3] and preparative chromatograms carried out on the aminoethylated chains according to the methods described in LEHMANN and HUNTSMAN [4]

Case Report

The *propositus* (R M) was a 17 year-old male who was first investigated at the age of 12 years because of recurrent attacks of jaundice and pigmenturia. On questioning he admitted to a long history of attacks of lassitude associated with pallor and precipitated by infections. On clinical examination apart from jaundice and a palpable spleen (3 cm) no other abnormality was found. The haematological findings were as follows: Hb 12.6 g/100 ml, PCV 40%, red cell count $4.4 \times 10^6/\text{mm}^3$, reticulocytes 9.0%, platelets $220,000/\text{mm}^3$, and WBC $7,500/\text{mm}^3$. The blood film showed a few irregularly contracted cells with a moderate degree of anisocytosis, polychromasia and basophilic stippling. Bone marrow smears showed normoblastic erythroid hyperplasia. Heinz bodies were detected after sterile incubation of the patient's red cells for 48 h. On heating a haemolysate a precipitate formed within 15 min. On analysis of the haemoglobins on starch gel electrophoresis at pH 8.6 a minor fraction was detected migrating cathodially to Hb A. The Hb A₂ level was slightly raised namely 3.9% (normal range 2.5–3.5%) and the Hb F was 1.7% (normal range up to 0.8%). The findings of other relevant investigations are given in table I.

Family history The family was investigated over 4 generations (fig 1). Four other members were found to be affected as judged by the presence of a haemolytic anaemia and the finding of a heat unstable haemoglobin. Also two other members were suspected to be affected on the clinical findings. The findings on this family are summarised in table II.

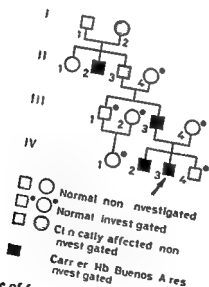


Fig 1 Pedigree of family heterozygous for Hb Buenos Aires

Table I Haematological and biochemical findings on patient R. M.

| | Patient | Normal |
|---|-----------|------------|
| Haemoglobin, g/100 ml | 12.6 | |
| Haematocrit, % | 40 | |
| Red cell count/mm ³ | 4,400,000 | |
| Reticulocyte count, % | 9.0 | |
| Platelet count/mm ³ | 220,000 | |
| White blood cell count/mm ³ | 7,500 | |
| Serum bilirubin, mg/100 ml | 1.6 | 0.2-0.7 |
| Haptoglobin, mg/100 ml | absent | 115 |
| Serum iron, µg/100 ml | 144 | 80-120 |
| Total iron binding capacity, µg/100 ml | 340 | 250-350 |
| Heinz bodies, 120 min incubation | negative | negative |
| Heinz bodies, 48 h incubation | positive | negative |
| Autohaemolysis, 48 h, % | 2.8 | 2.0 |
| Autohaemolysis, 48 h + glucose, % | 0.7 | 0.5 |
| Methaemoglobin, % | 2.4 | 0.7 |
| Brewer's test | positive | negative |
| GSH, mg/100 ml | 67.0 | 60.0-120.0 |
| GSH reductase, U/10 ¹⁰ red cells | 1.8 | 1.5-2.5 |
| G-6-PD, U/10 ¹⁰ red cells | 6.5 | 3.3-6.9 |
| P.K., U/10 ¹⁰ red cells | 9.0 | 4.0-8.0 |

Table II Clinical and Haematological findings of carriers in Hb Buenos Aires

| Patient ¹ | Sex | Haemoglobin
g/100 ml | Reticulocytes
% | Clinical findings |
|----------------------|-----|-------------------------|--------------------|--|
| I-2 | F | - | - | pallor, jaundice, dark urine |
| II-2 | M | 13.5 | 4.0 | jaundice, splenomegaly |
| II-3 | M | - | - | jaundice, splenomegaly |
| III-3 | M | 13.0 | 6.0 | jaundice, dark urine
general malaise,
splenectomy at 30 years
of age |
| IV-2 | M | 14.0 | 3.5 | jaundice, splenomegaly |
| IV-3 | M | 12.6 | 9.0 | propositus, jaundice,
pallor, dark urine,
general malaise,
splenomegaly |

¹ See figure 1

Table III Amino acid analysis of abnormal aminoethylated β Tp λ (83-95)

| Residue | Molar ratio | Expected molar ratio |
|---------|-------------|----------------------|
| Asp | | |
| Thr | 1.09 | |
| Ser | 1.78 | 1 |
| Glu | 1.87 | 2 |
| Gly | 1.14 | 1 |
| Ala | 1.00 | 1 |
| Leu | 1.03 | 1 |
| Phe | 2.02 | 1 |
| His | nil | 1 |
| Lys | 0.90 | 2 |
| AE Cys | | 1 |
| | 1.42 | 1 |
| | | 1 + 1 |

Table IV The tryptic peptide $\beta\lambda$ (83-85)

| Sequence No | 83 | 84 | 85 | 86 | 87 | 88 | 89 | 90 | 91 | 92 | 93 | 94 | 95 |
|-----------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Helical No | F1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | FG1 | 2 |
| Hb A | Gly | Thr | Phe | Ala | Thr | Leu | Ser | Glu | Leu | His | Cys | Asp | Lys |
| Hb Buenos Aires | Gly | Thr | Ser | Ala | Thr | Leu | Ser | Glu | Leu | His | Cys | Asp | Lys |
| | | | ↑ | | ↑ | | | ↑ | | | | | |

The peptide bonds broken with thermolysin are indicated with an arrow

The Amino Acid Substitution

The abnormal haemoglobin was isolated by precipitation with isopropanol and dissolved in 0.1 N HCl. The globin was reprecipitated by adding the solution to 2% acid acetone at -20 °C, and the α and β chains separated. The β chain was aminoethylated, filtered on Sephadex, lyophilised and then digested with trypsin. The preparative chromatograms showed the tryptic peptide $\beta\lambda$ (residues 83-95) having lower chromatographic mobility than normal. The peptide was eluted, digested with 6 N HCl for 24 h and its amino acid composition analysed. It was found that an expected residue of phenylalanine was missing and that an extra residue of serine was present (table III). The only possible explanation for this was that the normal β 85 (F1) phenylalanine was replaced by serine. However, since the size of β Tp λ prevents a perfect analysis, the peptides were digested with thermolysin which breaks it up at the positions indicated in table IV.

The thermolysis peptides were separated by electrophoresis at pH 6.4, and subsequent analysis on each confirmed that the phenylalanine at position 85 was replaced by serine. The total haemoglobin was fingerprinted and the normal and abnormal tryptic peptides βX were eluted separately but under the same conditions, and analysed. The molar ratio of the abnormal serine indicated that the abnormal haemoglobin comprised 45–50% of the total. This was a hitherto undescribed mutation, and has been called Hb Buenos Aires.

Discussion

The finding of Hb Buenos Aires now brings the total number of different unstable haemoglobins to 43 [5]. Unstable haemoglobins now represent the largest class of haemoglobin variants which result in disease in the heterozygous state [5]. The majority has been inherited as autosomal codominant genes, but some have arisen as a new mutation. They have been found in all parts of the world and in many ethnic groups. After Hb Buenos Aires had been reported [6], it was later found independently in America and called Hb Bryn Mawr [7].

Phenylalanine $\beta 85$ (F1) is present in the β -, γ - and δ -chains, but in the α -chain, this position is occupied by leucine. The reason why this particular mutation should cause the molecule to be unstable has been explained by M. F. PERUTZ from the examination of the atomic model of human haemoglobin. Residue $\beta 85$ is an internally sited residue which acts as a spacer between the F and III helical segments. If its bulky side chain is replaced by the smaller, hydrophilic side chain of serine, two things might happen. First the spacing factor would be lost, and secondly, there would be a tendency for water to be attracted into the normally hydrophobic haem pocket. Both of these factors would impart instability to the haemoglobin molecule.

The presence of this unstable haemoglobin causes a well compensated haemolytic anaemia and the patients have haemoglobin levels within the normal range. In many of the unstable haemoglobin disorders, this has been shown to be due to the high oxygen affinity of the haemoglobin [8].

The studies of BRADLEY *et al.* [7] have shown that this is the case for Hb Buenos Aires in that the whole cells and also the isolated haemoglo-

Table V Abnormalities produced by the substitution of phenylalanine residues of Hb A

| Variant | Chain | Substitution | Clinical disorder | Base change |
|--------------|----------|--------------------------|-------------------|-------------|
| Torino | α | CD1 Phe-Val | mild | U-G |
| Hammersmith | β | CD1 Phe-Ser | severe | U-C |
| Bucaresti | β | CD1 Phe-Leu | moderate | U-C |
| Christchurch | β | E15 Phe-Ser ¹ | moderate | U-C |
| Buenos Aires | β | F1 Phe-Ser ¹ | moderate | U-C |

¹ Phenylalanine is not invariant in this position. In the α -chain the site is occupied by leucine.

The substitution in Hb Buenos Aires (Phe→Ser) now brings the total number of substitutions of phenylalanine in the haemoglobin molecule to 5 (table V). It is of interest that all the variants are unstable, underlining the importance of this particular large amino acid for stability of haemoglobin. It is also noteworthy that the Phe→Ser mutation produces much more instability than the mutation Phe→Val, and Phe→Leu. This can be explained by the fact that serine has a smaller side chain, and also it is the only one which has hydrophilic properties.

Amongst the unstable haemoglobins which result from a single change in the triplet base codon for an amino acid, there are 19 examples of transversion mutations and 16 examples of transitions [5]. This deviation from the expected 2:1 ratio has been pointed out before [9-11] and used as evidence that viable mutations in the haemoglobin molecule may not be occurring at random. This is supported in part by examination of phenylalanine mutations. Single-point mutations in the codons for phenylalanine, UUU, UUC, could theoretically occur 12 times: 8 transversions and 4 transitions. Therefore, it is of interest that on the 5 occasions where base changes are observed, only 1 transversion is found but 4 transitions (table V). It is unlikely that the explanation is merely that the amino acids which result from transition mutation, leucine and serine, result in more instability (thus easier detection), than those which would result from transversion mutations valine, tyrosine, cysteine, and isoleucine. However, since substitutions by the last three amino acids have not been reported, this remains speculation.

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Deutsche und Österreichische Gesellschaft für Hämatologie

Vom 21 bis 23 März 1974 finden in Wien als gemeinsame Tagung die 17. Jahrestagung der Deutschen Gesellschaft für Hämatologie und die 4. Jahrestagung der Österreichischen Gesellschaft für Hämatologie statt. Präsident: Prof. Dr. E. DEUTSCH, I. Medizinische Universitätsklinik Wien. Sekretär: Dr. N. HONETZ, I. Medizinische Universitätsklinik Wien.

Thema der Tagung: Die Knochenmarkinsuffizienz (unter Ausschluss der malignen Erkrankungen).

Vorläufiges wissenschaftliches Programm: Pathologie und pathophysiologische Grundlagen. 1. Die hämopoetische Stammzelle, 2. Physiologie und Pathologie der Knochenmarkmatrix, 3. Anwendung von Kulturverfahren, 4. Proliferationskinetik bei Panmyelopathien, 5. Humorale Regulation der Hämoese, insbesondere bei Knochenmarkinsuffizienz, 6. Zytologie und Zytochemie, 7. Die klinische Histologie der Knochenmarkinsuffizienz, Pathogenese und Klinik. 1. Pathogenese und Klinik der Panmyelophthase, 2. Isolierte aplastische Anämie, 3. Agranulozytose, 4. Besonderheiten der Knochenmarkinsuffizienz bei Kindern, 5. Enzymdefekte bei Knochenmarkinsuffizienz, 6. Toxikologie und Genetik, 7. Immunologie. Therapie. 1. Medikamentöse Beeinflussung der Knochenmarkinsuffizienz, 2. Besonderheiten der Infektionstherapie, 3. Probleme der Infektionsprophylaxe, 4. Möglichkeiten der Behandlung der hämorrhagischen Diathesen bei Knochenmarkinsuffizienz, 5. Knochenmarktransplantation. Round Table-Gespräch: Die Rolle der Milz für die Entstehung der Knochenmarkinsuffizienz.

Kongresssekretariat: Intercongress, Stadiongasse 6-8, A-1010 Wien (Österreich).

Journées d'Hématologie radioisotopique, Montpellier

Organisées par le Laboratoire des Radioéléments de l'Institut d'Hématologie de Montpellier, ces Journées auront lieu le 17 et 18 avril 1974 au Centre de Transfusion Sanguine, Avenue Emile Jeanbrau, 34-Montpellier, France.

Les thèmes à l'ordre du jour seront les suivants: 1. Les protéines plasmatiques. 2. La cinétique du fer et de l'érythropoïèse. 3. Les tests *in vitro* à intérêt hématologique.

Pour tous renseignements s'adresser au Dr. A. WAGNER, Institut d'Hématologie, BP no 1213, 34010-Montpellier, France.

Index rerum ad Vol. 50

Bearbeitet von ■ BOEHM, Basel

(B) = Book reviews – Buchbesprechungen – Livres nouveaux

- Acetylsalicylic acid, v Myeloproliferative disorders
- Actinomycin D, study of the effect on the thrombocytopoiesis of mice, using ⁷⁵Se-labelled methionine, 168
- Acquired factor IX deficiency (A report of two cases), 305
- Adenosindiphosphate (= ADP) defect in platelets, v EHLERS-DANLOS syndrome
- Adhesiveness of platelets v Thrombocytopathy
- ADP, v Adenosindiphosphate
- Aggregation of platelets, v EHLERS-DANLOS syndrome, Thrombocytopathy
- Agranular blasts, v Metamorphosis of myeloid leukaemia
- Agranulocytosis, v Leukocyte kinetics
- Alkaline phosphatase, v Phosphatase, alkaline
- Anaemia, aplastic, studies on bone marrow transplantation = experimental ³²P induced aplastic anaemia after conditioning with antilymphocyte serum, 193
- Anaemia, mediterranean, v Thalassaemia
- Anesthetized mice, effects of irradiation on the haematopoietic tissues, 50
- Antibody, the prevalence of Australia antigen and antibody in haemophilia, 293
- Anticoagulant, circulating, v Factor IX
- Antigen, the prevalence of Australia antigen and antibody in haemophilia, 293
- Antihæmophilic globulin ■ (= AHG B), v Factor IX
- Antilymphocyte serum, studies on bone marrow transplantation in experimental ³²P induced aplastic anaemia after conditioning with antilymphocyte serum, 193
- Aplasia of bone marrow, v Leukocyte kinetics
- Aplastic anaemia, studies on bone marrow transplantation in experimental ³²P-induced aplastic anaemia after conditioning with antilymphocyte serum, 193
- Arabs haemoglobin C in Arabs in Kuwait, 112
- Asparaginase, the effect of L-asparaginase on DNA and RNA synthesis by lymphoblasts of acute lymphocytic leukaemia, 269
- , mechanism of action of L-asparaginase on the cell cycle and growth in acute lymphoblastic leukaemia, 257
- Aspirin, v Myeloproliferative disorders
- Australia antigen and antibody, prevalence in haemophilia, 293
- Autoradiography, v Asparaginase, Disopropylfluorophosphate, Rat leukocyte cultures
- Basic immunogenetics, 191 (B)
- Beta-thalassaemia, v Thalassaemia

- Bindegewebskrankung, v EHLERS DAN-
 LOS syndrome
 Blastie transformation, v Metamorphosis
 of myeloid leukaemia
 Bleeding disorders, v Blood coagulation
 Bleeding time, prolonged; congenital
 thrombocytopathy (platelet factor 3 de-
 fect) with prolonged bleeding time but
 normal platelet adhesiveness and aggre-
 gation, 116
 Blood coagulation, v Australia antigen,
 Bleeding time, Factor IX, EHLERS-DAN-
 LOS syndrome, Fibrinogenspalprodukt-
 te, Hypoproconvertinaemia
 Blood group and tissue mosaicism in a
 Natal Indian woman, 299
 Blood transfusion, v Australia antigen
 Blutgruppe, v Blood group
 Blutplättchen, v Cyclophosphamid,
 EHLERS DANLOS syndrome, Platelet ag-
 gregation, Platelet defect, Thrombocy-
 thaemia, Thrombocytopathy, Thrombo-
 cytopoiesis
 B lymphocytes, v Surface markers
 Bone marrow culture, v Asparaginase
 Bone marrow, v Cyclophosphamid, Irra-
 diation, Neutrophils of bone marrow
 Bone marrow transplantation in experi-
 mental $\alpha\beta$ induced aplastic anaemia
 after conditioning with antilymphocyte
 serum, studies, 193
 Book reviews, 64 (B), 191-192 (B)
 Buchbesprechungen, 64 (B) 191-192 (B)
 Canavalia ensiformis, v Concanavalin A
 Carrier proteins of folic acid, v Folic acid
 Cell cultures, v Asparaginase, Rat leuko-
 cyte cultures
 Cell cycle, mechanism of action of L-aspar-
 aginase on the cell cycle and growth in
 acute lymphoblastic leukaemia, 257
 Cell release from bone marrow, v Leuko-
 cyte kinetics
 Chimera, blood group chimera, v Blood
 group (mosaicism)
 Christmas factor, v Factor IX
 Chromatographie; säulenchromatographi-
 sche Anreicherung von DNA-Polymer-
 ase-Aktivitäten bei Leukämie, 200
 Chromatography, gel chromatography, v.
 Folic acid
 Chromosome studies in paroxysmal noc-
 turnal haemoglobinuria, 350
 Chromosomes, v Blood group (mosaicism)
 Chronic myeloid leukaemia, 64 (B)
 Coagulation of blood, v Australia antigen,
 Bleeding time, EHLERS DANLOS syndro-
 me, Factor IX, Fibrinogenspalprodukt-
 te, Hypoproconvertinaemia
 Colony forming units, a comparison of the
 three *in vivo* assays for haemopoietic
 stem cells, 9
 Comparative haematology, v Eosinophile
 Concanavalin A, ultrastructural features of
 phythaemagglutinin and concanavalin
 A Responsive lymphocytes in chronic
 lymphocytic leukaemia, 129
 Congenital hypoproconvertinaemia (factor
 VII deficiency) (A report of two cases
 belonging to two different kindreds), 228
 Congenital thrombocytopathy (platelet fac-
 tor 3 defect) with prolonged bleeding
 time but normal platelet adhesiveness
 and aggregation, 116
 Congress, v Hämatologie
 Connective tissue disease, v EHLERS-
 DANLOS syndrome
 Convertin, v Hypoproconvertinaemia
 Corticosteroids, treatment of terminal
 metamorphosis of chronic granulocytic
 leukaemia with corticosteroids and vin-
 cristine, 1
 Cryoglobulinaemia, v Pyroglobulinaemia
 2-¹⁴C-Thymidintriphosphat, v Polymerase
 Cuban family, haemoglobin D Punjab in a
 Cuban family and its interaction with
 haemoglobin S, 315
 Culture des cellules, v Asparaginase, Rat
 leukocyte cultures
 Cyclophosphamid, Einfluss auf Enzyme der
 Megakaryozyten, Untersuchungen, 217
 Cytochemistry, v Asparaginase, Cyclo-
 phosphamid

- Cytophotometry, v Erythropoese
- Cytospectrophotometry, v Asparaginase
- Cytotoxic agents, v Haemopoietic stem cells
- DANLOS-ERLERS syndrome, platelet defect in a case, 238
- Desoxyribonucleinsäure, v DNA
- Deutsche und Österreichische Gesellschaft für Hämatologie, 17, resp 4 Jahrestagung (Wien, 21-23 März 1974), 364
- DFP, v Diisopropylfluorophosphate
- Dialysis, v Haemodialysis
- Diffusion chambers, v Rat leukocyte cultures
- Diisopropylfluorophosphate (=DFP) (radioactive), uptake by granulocytopenic cells in chronic myeloid leukaemia and in normal individuals, 143
- Diphenylhydantoin, v Folic acid
- Dispermy, v Blood group (mosaicism)
- Distamycin, v Polymerase
- DNA (= Desoxyribonucleinsäure)-Polymerase-Aktivitäten bei Leukämie, säulenchromatographische Anreicherung, 200
- DNA (= Desoxyribonucleic acid) and RNA (= Ribonucleic acid) synthesis by lymphoblasts of acute lymphocytic leukaemia, effect of L-asparaginase, 269
- Double fertilization, v Blood group (mosaicism)
- Double population of erythrocytes, v Blood group (mosaicism)
- EAC rosettes, v Lymphocyte surface markers
- ERLERS-DANLOS syndrome, platelet defect in a case 238
- Eidechse, Erythropoese, v *Lacerta muralis*
- Einschlüsse, neuartige, im Ergastoplasma peripherer Lymphozyten bei Virusinfekt, 245
- Electron microscope, v Concanavalin A, Einschlüsse, neuartige (Lymphozyten) Eosinophile, Pyknocytosis, Ropocytosis
- Electrophoresis, v Glucose-6-phosphate dehydrogenase, Haemoglobin D Punjab, Haemoglobin H, Haemoglobin O Indonesia, Pyroglobulinaemia
- Emergence time of mature marrow neutrophils, granulocyte alkaline phosphatase activity, a measure? 19
- Endoplasmatisches Retikulum, v Einschlüsse, neuartige
- Enzyme der Megakaryozyten, Untersuchungen über den Einfluss des Cyclophosphamids, 217
- Eosinophile, spezifische Mikrogranula in Eosinophilen (Eine vergleichende elektronenmikroskopische Untersuchung an verschiedenen Säugern zur Charakterisierung einer besonderen Granulationsform bei eosinophilen Granulozyten), 92
- E (= Erythrocyte) rosettes, v Lymphocyte surface markers
- Erythroblasten, Synthese von Hämoglobin, RNS und Proteinen in der normalen Erythropoese, 340
- Erythrocyte rosettes, v Lymphocyte surface markers
- Erythrocytes, pyknocytosis in heat-stroke, 249
- Erythrocytes with pseudopodia, ropocytosis in a patient with acute lymphoblastic leukaemia, 44
- Erythrocytes (two populations), v Blood group (mosaicism)
- Erythrodermia, mycosis fungoides, skin reticulosis - autonomous disorders of the monocytopenic macrophage system? 329
- Erythroid repopulation, a comparison of the three *in vivo* assays for haemopoietic stem cells, 9
- Erythromyelosis, v Panmyelose
- Erythropoese, Synthese von Hämoglobin, RNS und Proteinen in der normalen Erythropoese, 340
- Erythropoëse chez *Lacerta muralis* (Laurenti), morphologie, 56
- Factor I, v Fibrinogenspaltprodukte

- ... deficiency
 ... (A)
 ... belonging to two
 ... Christmas factor)
 ... (A report of two
 ... , Platelet factor
 ... group (mosaicism),
 ... VII, Haemoglobin
 ... C, Haemo-
 ... O Indonesia,
 ...
 ... Concanavalin A, Ein
 ... (Lymphozyten)
 ... Pyknocytosis, Ropalcocy-
 ...
 ... Megakaryozyten, Untersu-
 ... den Einfluss des Cyclo-
 ... 217
 ... Asparaginase, Glucose-6-
 ... dehydrogenase Polymerase
 ... double, v Blood group (mo-
 ...
 ... globin, hereditary persistence,
 ... in a Turkish child
 ...
 ... Produkte, Viskositätsunter-
 ... 75
 ... , v Concanavalin A, Ein
 ... (Lymphozyten)
 ... Pyknocytosis, Ropalcocy-
 ...
 ... v Haemoglobin D Punjab
 ... O Indonesia
 ... v Fetal
 ... acid, binding to serum proteins (I
 ... the effect of pregnancy) 85
 ... (II The effect of diphenylhyd-
 ... treatment and of various diseases
 284
 Gel chromatography, v Folic acid
 Genetics, basic immunogenetics, 191 (B)
 -, v Fanulies, Haemoglobinopathies, H
 redopathies
 Gesellschaft, Deutsche und Österreichische
 Gesellschaft für Hämatologie, 17, resp
 4 Jahrestagung (Wien, 21-23 März
 1974), 364
 Ghana glucose 6-phosphate dehydrogen-
 ase electrophoresis in *Ghanaians* with
 AA and SS haemoglobin, 105
 Glucose-6-phosphate dehydrogenase (= G-
 6-PD) electrophoresis in *Ghanaians* with
 AA and SS haemoglobin, 105
 G 6 PD, v Glucose-6-phosphate dehydro-
 genase
 Granular blasts v Metamorphosis of mye-
 loid leukaemia
 Granulocyte alkaline phosphatase activity
 a measure of the emergency time of ma-
 ture marrow neutrophils? 19
 Granulocytic leukaemia, chronic, treat-
 ment of terminal metamorphosis of
 chronic granulocytic leukaemia with cor-
 ticosteroids and vincristine, 1
 Granulocytic repopulation, a com-
 on of the three *in vivo* assays f
 poietic stem cells, 9
 Granulocytopoiesis, v Leuk
 Granulocytopoietic cells in c
 temia and in no
 of disopropyl
 Pregnancy
 ins, v Bl
 hanism of
 the cell
 blastic

- Haemoglobin Buenos Aires β 85 (F 1) phe \rightarrow ser, a new unstable haemoglobin, 357
- Haemoglobin C in *Arabs in Kuwait*, 112
- Haemoglobin D Punjab in a *Cuban* family and its interaction with haemoglobin S, 315
- Haemoglobin F, hereditary persistence of fetal haemoglobin and β -thalassaemia in a *Turkish* child, 124
- Haemoglobin H β -thalassaemia, 184
- Haemoglobin, new, unstable haemoglobin Buenos Aires, β 85 (F 1) phe \rightarrow ser, 357
- Haemoglobin O Indonesia (all 16 glutamic acid \rightarrow lysine) in an *Iranian* family, 30
- Haemoglobin S, haemoglobin D Punjab in a *Cuban* family and its interaction with haemoglobin S, 315
- , v Glucose-6-phosphate dehydrogenase
- Hämoglobinbildung Synthese von Hämoglobin, RNS und Proteinen in der normalen Erythropoese, 340
- Haemoglobinopathies, serum haemopexin concentration in patients with various haemoglobinopathies (Effect of splenectomy), 149
- Haemoglobinopathies, v Glucose-6-phosphate dehydrogenase, Haemoglobin Buenos Aires, Haemoglobin C, Haemoglobin D Punjab, Haemoglobin H, Haemoglobin O Indonesia
- Haemoglobinuria, paroxysmal nocturnal haemoglobinuria (PNH) (Ser haematol Vol V/1), 192 (B)
- Haemoglobinuria, paroxysmal, nocturnal, chromosome studies, 350
- Haemodialysis, some aspects of leukocyte behaviour in haemodialysis, 223
- Haemopexin, serum haemopexin concentration in patients with various haemoglobinopathies (Effect of splenectomy) 149
- Haemophilia, the prevalence of Australia antigen and antibody in haemophilia, 293
- Haemopoietic stem cells, a comparison of the three *in vivo* assays for haemopoietic stem cells, 9
- Hb A, v Haemoglobin A
- Hb C, v Haemoglobin C
- Hb D, v Haemoglobin D Punjab
- Hb F, v Haemoglobin F
- Hb H, v Haemoglobin H
- Hb O v Haemoglobin O Indonesia
- Hb S, v Haemoglobin S
- 3 H-diisopropylfluorophosphate, v Diisopropylfluorophosphate
- Heat-stroke, pyknocytosis in heat-stroke, 249
- Hematologie, Journées d'hématologie radioisotopique, (Montpellier, 17 et 18 avril 1974), 364
- Hemoglobin H β -thalassaemia, 184
- Hem, Hém, v Haem
- Hereditary persistence of fetal haemoglobin and β -thalassaemia in a *Turkish* child, 124
- Heredopathies, v EHRLER-DANLOS syndrome, Factor VII, Haemoglobin C, Haemoglobin H, Haemoglobin O Indonesia, Hereditary, Hypoproconvertin aemia
- Histochemistry, v Asparaginase, Cyclophosphamid
- Hitzschlag, v Heat-stroke
- HODGKIN's disease, the value of laparotomy and splenectomy in the staging of 56 patients with HODGKIN's disease, 321
- HODGKIN, morbus HODGKIN, v Syndrome de RICHTER
- 3 H thymidine, v Asparaginase, Concavalin A, Monocytopenia, Rat leukocyte cultures
- 3 H-uridine, v Asparaginase
- IgG-myeloma, v Pyroglobulinaemia
- IgM, v Pyroglobulinaemia
- Immunodiffusion, v Haemopexin
- Immuno-electro-osmiodiffusion, v Australia antigen
- Immunoelectrophoresis, v Pyroglobulinaemia
- Immunofluorescence, v Surface markers
- Immunogenetics, basic immunogenetics, 191(B)

- Factor VII (= Proconvertin) deficiency
Congenital hypoproconvertinaemia (A
report of two cases belonging to two
different kindreds), 228
- Factor IX (= AHG II = Christmas factor)
deficiency, acquired (A report of two
cases), 305
- Factor 3, v Platelet defect, Platelet factor
3 defect
- Families, v Blood group (mosaicism),
Cuban family, Factor VII, Haemoglobin
Buenos Aires, Haemoglobin C, Haemo-
globin H-, Hemoglobin O Indonesia,
Hypoproconvertinaemia
- Feinstruktur, v Concanavalin A, Ein-
schlüsse, neuartige (Lymphozyten),
Eosinophile, Pyknocytosis, Ropalocy-
tosis
- Fermente der Megakaryozyten, Untersu-
chungen über den Einfluss des Cyclo-
phosphamids, 217
- Ferments, v Asparaginase, Glucose-6-
phosphate dehydrogenase, Polymerase
- Fertilization, double, v Blood group (mo-
saicism)
- Fetal haemoglobin, hereditary persistence,
and a thalassaemia in a *Turkish* child,
124
- Fibrinogenspaltprodukte, Viskositätsunter-
suchungen, 75
- Fine structure, v Concanavalin A, Ein-
schlüsse, neuartige (Lymphozyten),
Eosinophile, Pyknocytosis, Ropalocy-
tosis
- Fingerprints, v Haemoglobin D Punjab,
Haemoglobin O Indonesia
- Foetal, v Fetal
- Folic acid, binding to serum proteins (I
The effect of pregnancy), 85
- - - (II The effect of diphenylhydantoin
treatment and of various diseases),
284
- Gel chromatography, v Folic acid
- Genetics, basic immunogenetics, 191 (B)
-, v Families, Haemoglobinopathies, He-
redopathies
- Gesellschaft, Deutsche und Österreichische
Gesellschaft für Hämatologie, 17, resp
4 Jahrestagung (Wien, 21-23 März
1974), 364
- Ghana glucose-6-phosphate dehydrogen-
ase electrophoresis in *Ghanaians* with
AA and SS haemoglobin, 105
- Glucose-6-phosphate dehydrogenase (= G-
6-PD) electrophoresis in *Ghanaians* with
AA and SS haemoglobin, 105
- G-6-PD, v Glucose-6-phosphate dehydro-
genase
- Granular blasts, v Metamorphosis of mye-
loid leukaemia
- Granulocyte alkaline phosphatase activity
a measure of the emergency time of ma-
ture marrow neutrophils? 19
- Granulocytic leukaemia, chronic, treat-
ment of terminal metamorphosis of
chronic granulocytic leukaemia with cor-
ticosteroids and vincristine, 1
- Granulocytic repopulation, a comparison
of the three *in vivo* assays for haemo-
poietic stem cells, 9
- Granulocytopenia, v Leukocyte kinetics
- Granulocytopenic cells in chronic myeloid
leukaemia and in normal individuals,
uptake of diisopropylfluorophosphate,
143
- Grossesse, v Pregnancy
- Groupes sanguins, v Blood group
- Growth, mechanism of action of L-aspar-
aginase on the cell cycle and growth in
acute lymphoblastic leukaemia, 257
- Hämatologie, Deutsche und Österreichi-
sche Gesellschaft für Hämatologie, 17,
resp 4 Jahrestagung (Wien, 21-23
März 1974), 364
- Hämatologie, vergleichende, v Eosinophile
- Haematologie, Journées d'hématologie ra-
dioisotopique (Montpellier, 17 et 18
avril 1974), 364
- Haematopoietic tissues of anaesthetized
mice, effects of irradiation, 50
- Haemoglobin A, v Glucose-6-phosphate
dehydrogenase

- Leukopenia, v Haemodialysis
- Lézard, érythropoïèse chez *Lacerta muralis* (Laurenti), morphologie, 56
- Libri, ㊦ (B), 191-192 (B)
- Lien, v Splenectomy, Splenic function
- Livres nouveaux, ㊦ (B), 191-192 (B)
- Lizard erythropoiesis, v *Lacerta muralis*
- Lymphatic leukaemia, chronic, v Syndrome de Richter
- Lymphoblastic leukaemia, acute, mechanism of action of L-asparaginase on the cell cycle and growth in acute lymphoblastic leukaemia, 257
- -, ropalocytosis in a patient with acute lymphoblastic leukaemia, 44
- Lymphoblasts of acute lymphocytic leukaemia, effect of L-asparaginase on their DNA and RNA synthesis, 269
- Lymphocyte surface markers in lymphoproliferative disorders, 275
- Lymphocyten, neuartige Einschlüsse im Ergastoplasma peripherer Lymphocyten bei Virusinfekt, 245
- Lymphocytes, antilymphocyte serum studies on bone marrow transplantation in experimental ³²P induced aplastic anaemia after conditioning with antilymphocyte serum, 193
- Lymphocytic leukaemia, acute, the effect of L-asparaginase on DNA and RNA synthesis by lymphoblasts of acute lymphocytic leukaemia, 269
- Lymphocytic leukaemia, chronic, ultrastructural features of phythaemagglutinin and concanavalin A Responsive lymphocytic leukaemia, 129
- Lymphogranulomatosis maligna, the value of laparotomy and splenectomy in the staging of 36 patients with Hodgkin's disease, 321
- Lymphoid tissues, v Irradiation
- Lymphoproliferative disorders, lymphocyte surface markers in lymphoproliferative disorders, 275
- Macrophages, erythrodermia, mycosis fungoides, skin reticulosis - autonomous disorders of the monocytopoietic macrophage system? 329
- Mammalia, v Eosinophile
- Mature marrow neutrophils, v Neutrophils of bone marrow
- Maus, v Mice
- Mediterranean anaemia, v Thalassaemia
- Medulla osseum, v Bone marrow
- Megakaryocytic myelosis, v Panmyelosis
- Megakaryozyten, Untersuchungen über den Einfluss des Cyclophosphamids auf Enzyme der Megakaryozyten, 217
- Membran surface immunoglobulins, v Surface markers
- Metamorphosis of myeloid leukaemia, treatment of terminal metamorphosis of chronic granulocytic leukaemia with corticosteroids and vincristine, 1
- Methionine (¹²⁵Se-methionine), v Actinomycin D
- Mice, anaesthetized, effects of irradiation on the haematopoietic tissues, 50
- Mice, study of the effect of actinomycin D on the thrombocytopoiesis of mice, using ¹²⁵Se-labelled methionine, 168
- Microautoradiography, v Autoradiography
- Microscope électronique, v Concanavalin A, Einschlüsse, neuartige (Lymphocyten), Eosinophile Pyknocytosis, Ropalocytosis
- Mikrogranula, spezifische, in Eosinophilen (Eine vergleichende elektronenmikroskopische Untersuchung an verschiedenen Säugern zur Charakterisierung einer besonderen Granulationsform bei eosinophilen Granulozyten) 92
- Milz, v Splenectomy, Splenic function
- Moelle osseuse v Bone marrow
- Monocytic leukaemia, Übergang einer Polycythaemia vera in eine akute Monocytenleukämie, 36
- Monocytopenia, disorders, erythrodermia, mycosis fungoides, skin reticulosis - autonomous disorders of the monocytopoietic macrophage system? 329
- Monozytenleukämie, Übergang einer Poly-

- Immunoglobulins, v. Pyroglobulinaemia, Surface markers
- Inclusions, novel, v. Neuartige Einschlüsse (Lymphozyten)
- Indian woman (in *Natal*), v. Blood group (mosaicism)
- Infection splenic function and infection in sickle cell anaemia, 154
- Iranian family, haemoglobin O Indonesia (a 116 glutamic acid→lysine) in an Iranian family, 30
- Irradiation, effects on the haematopoietic tissues of anaesthetized mice, 50
- Isotope, radioaktive, v. Actinomycin D, Asparaginase, Concanavalin A, Diisopropylfluorophosphate, Hématologie radioisotopique, Monocytopoiesis, Polycythaemia vera, Polymerase, Radiophosphorus, Sickle cell anaemia
- Jackbohne, v. Concanavalin A
- Journées d'hématologie radioisotopiques, (Montpellier, 17 et 18 avril 1974), 364
- Karyokinetics, v. *Lacerta muralis*
- Karyometrie, v. Erythropoese
- Karyotype, v. Blood group (mosaicism)
- Paroxysmal nocturnal haemoglobinuria
- Knochenmark v. Bone marrow
- Koloniebildung, v. Colony forming units
- Kongenital, v. Congenital
- Kongress, v. Hématologie
- Kuwait*, haemoglobin C in *Arabs* in *Kuwait*, 112
- Lacerta muralis* morphologie de l'érythropoïèse chez *Lacerta muralis* (Laurenti), 56
- Laparotomy and splenectomy in the staging of 56 patients with HODGKIN's disease, their value, 321
- L asparaginase v. Asparaginase
- Leucémie lymphoïde chronique, v. Syndrome de RICHTER
- Leukaemia following polycythaemia, v. Leukaemia, monocytic
- Leukaemia, granulocytic, chronic, treatment of terminal metamorphosis of chronic granulocytic leukaemia with corticosteroids and vincristine, 1
- Leukaemia, lymphatic, chronic, v. Syndrome de RICHTER
- Leukaemia, lymphoblastic, acute, mechanism of action of L-asparaginase on the cell cycle and growth in acute lymphoblastic leukaemia, 257
- — —, reticulocytosis in a patient with acute lymphoblastic leukaemia, 44
- Leukaemia, lymphocytic, acute, the effect of L-asparaginase on DNA and RNA synthesis of lymphoblasts of acute lymphocytic leukaemia, 269
- Leukaemia, lymphocytic, chronic, ultrastructural features of phytohemagglutinin and concanavalin A responsive lymphocytes in chronic lymphocytic leukaemia, 129
- Leukaemia, monocytic acute, Übergang einer Polycythaemia vera in eine akute Monozytenleukämie, 36
- Leukaemia, myelogenous, v. Myeloproliferative disorders
- Leukaemia, myeloid, chronic, 64 (B)
- Leukaemia, myeloid, chronic, diisopropyl fluorophosphate uptake by granulocytopoietic cells in chronic myeloid leukaemia, and in normal individuals, 134
- Leukaemia therapy, v. Asparaginase
- Leukaemia, v. Panmyelose
- Leukämie, 64 (B)
- Leukämie, säulenchromatographische Anreicherung von DNA-Polymerase-Aktivitäten bei Leukämie, 200
- Leukocyte, alkaline phosphatase, v. Leukocyte kinetics
- Leukocyte behaviour in haemodialysis, some aspects, 223
- Leukocyte cultures (rat leukocytes), ribonucleic acid and phytohaemagglutinin on rat leukocyte cultures within diffusion chambers, 162
- Leukocyte kinetics, granulocyte alkaline phosphatase activity a measure of the emergence time of mature marrow neutrophils? 19

- Pregnancy, bindings of folic acid to serum proteins (I The effect of pregnancy) 85
 - - - -, (II The effect of diphenylhydantoin treatment and of various diseases), 284
- Proconvertin (= Factor VII) v Hypoproconvertinaemia
- Proliferation rates, v Haemopoietic stem cells
- Protein Bildung, Synthese von Hämoglobin, RNS und Proteinen in der normalen Erythropoese, 340
- Protein synthesis, v Actinomycin D
- Proteins, serum proteins, binding of folic acid (I The effect of pregnancy) 85
 - - - -, (II The effect of diphenylhydantoin treatment and of various diseases), 284
- Pseudopodia' of erythrocytes, v Ropalcytosis
- ³²P-therapy, v Polycythaemia vera
- Pyknocytosis in heat-stroke, 249
- Pyroglobulinaemia (A report of eight patients with associated paraproteinaemia) 65
- Radiation, v Irradiation
- Radioisotopes, Journées d'hématologie radioisotopique, (Montpellier, 17 et 18 avril 1974), 364
- Radio-phosphorus, v ³²P induced aplastic anaemia, Polycythaemia vera
- Radio-selenium, v Actinomycin D
- Rasterelektronenmikroskop, v Pyknocytosis
- Rat bone marrow, v Cyclophosphamid
- Rat leukocyte cultures, ribonucleic acid and phytohaemagglutinin on rat leukocyte cultures within diffusion chambers 162
- Rate, v Splenectomy, Splenic function
- Repopulation, v Haemopoietic stem cells
- Réticulopathie maligne (réticulosarcoma ou maladie de HOOGKRO) v Syndrome de RICHTER
- Reticulosarcoma, v Syndrome de RICHTER
- Reticulosis of the skin, erythrodermia, mycosis fungoides - autonomous disorders of the monocytopenic macrophage system? 329
- Reviews, transplantation reviews (Vol 11 and 12) 191 (B)
- Ribonucleic acid (= RNA) and phytohaemagglutinin on rat leukocyte cultures within diffusion chambers, 162
- Ribonucleic acid, v RNA, RNS
- RICHTER's syndrome (Rapport de quatre observations = essais de démemberment), 213
- Rifamycin, v Polymerase
- RNA (= Ribonucleic acid) and DNA (= Desoxyribonucleic acid) synthesis by lymphoblasts of acute lymphocytic leukaemia, effect of L-asparaginase, 269
- RNS (= Ribonukleinsäure), Synthese von Hämoglobin, RNS und Proteinen in der normalen Erythropoese, 340
- Ropalcytosis in a patient with acute lymphoblastic leukaemia, 44
- Rosettes, E rosettes, EAC rosettes, v Lymphocyte surface markers
- Rough surfaced endoplasmic reticulum, v Einschlüsse, neuartige (Lymphozyten)
- Rubeola, neuartige Einschlüsse im Ergastoplasma peripherer Lymphozyten bei Virusinfekt, 245
- Säugetiere, v Eosinophile
- Säulenchromatographische Anreicherung von DNA Polymerase-Aktivitäten bei Leukämie, 200
- Scanning electron microscope v Pyknocytosis
- Schwangerschaft, v Pregnancy
- Schwertbohne, v Concanavalin A
- ⁷⁵Se-methionine, v Actinomycin D
- Serum haemopexin concentration in patients with various haemoglobinopathies, 149
- Serum proteins, binding of folic acid (I The effect of pregnancy), 85
 - - - -, (II. The effect of diphenylhydantoin treatment and of various diseases), 284

- Sickle cell disease, v Glucose-6-phosphate dehydrogenase Haemopexin
- Sickle cell Hb D disease, v Haemoglobin D Punjab
- sIg, v Surface markers
- Skin reticulosis, erythrodermia, mycosis fungoides - autonomous disorders of the monocytopenic macrophage system? 329
- Society, v Gesellschaft
- Souris, v Mice
- Spleen colonies, v Irradiation
- Spleen colony forming units, v Colony forming units
- Splenectomy and laparotomy in the staging of 56 patients with HODGKIN's disease, their value, 321
- Splenectomy, serum haemopexin concentration in patients with various haemoglobinopathies (Effect of splenectomy) 149
- , v Aplastic anaemia
- Splenic function and infection in sickle cell anaemia, 154
- Spontaneous platelet aggregation in myeloproliferative disorders (A preliminary study), 25
- Staging in HODGKIN's disease, the value of laparotomy and splenectomy in the staging of 56 patients with HODGKIN's disease, 321
- Statistische Auswertung, v Concanavalin A, Diisopropylfluorophosphate, Glucose-6-phosphate dehydrogenase, Monocytopenia, Platelet aggregation, Rat leukocyte cultures, Sickle cell disease
- Stem cells, a comparison of the three *in vivo* assays for haemopoietic stem cells 9
- Strahlenwirkung, v Irradiation
- Submicroscopical structure, v Concanavalin A, Einschlüsse, neuartige (Lymphozyten) Eosinophile Pyknocytosis, Ropalocytosis
- Surface markers, lymphocyte surface markers in lymphoproliferative disorders, 275
- Syndrome de RICHTER (Rapport de quatre observations et essai de démembrement), 213
- $^{99}\text{Tc}^m$ sulfur colloid, v Sickle cell anaemia
- Technetium ($^{99}\text{Tc}^m$) sulfur colloid, v Sickle cell anaemia
- Terminal metamorphosis, v Metamorphosis of myeloid leukaemia
- Thalassaemia, haemoglobin H β -thalassaemia, 184
- , hereditary persistence of fetal haemoglobin and β -thalassaemia in a Turkish child, 124
- , v Haemopexin
- Therapy, v Asparaginase, Granulocytic leukaemia
- Thrombocytes, v Cyclophosphamid, Platelet aggregation, Platelet defect, Thrombocythaemia, Thrombocytopathy, Thrombocytopoiesis
- Thrombocythaemia, v Myeloproliferative disorders
- Thrombocytopathy, congenital (platelet factor 3 defect), with prolonged bleeding time but normal platelet adhesiveness and aggregation, 116
- Thrombocytopoiesis of mice, study of the effect of actinomycin D, using ^{75}Se -labelled methionine, 168
- Thrombopathy, v EHLERS DANLOS syndrome
- Thymidine (^3H thymidine) v Asparaginase, Concanavalin A, Monocytopenia, Rat leukocyte cultures
- Thymidintriphosphat ($2\text{-}^{14}\text{C}$ -Thymidintriphosphat), v Polymerase
- T lymphocytes, v Surface markers
- Tissue mosaic, blood group and tissue mosaicism in a Natal Indian woman, 299
- Transformation of leukaemic lymphocytes v Concanavalin A
- Transfusion, blood transfusion, v Australia antigen
- Transplantation reviews (Vol 11 and 12), 191 (B)
- Transplantation, studies on bone marrow transplantation in experimental ^{32}P in-

- duced aplastic anaemia after conditioning with antilymphocyte serum, 193
- Treatment of terminal metamorphosis of chronic granulocytic leukaemia with corticosteroids and vincristine, 1
- Tritium, v ^3H
- Turkish* child, hereditary persistence of fetal haemoglobin and β -thalassaemia in a *Turkish* child 124
- Ultrastructure, v Einschlüsse, neuartige (Lymphozyten), Eosinophile, Pyknocytosis, Ropalcytosis, Ultrastructural
- Ultrastructural features of phytohaemagglutinin and concanavalin A Responsive lymphocytes in chronic lymphocytic leukaemia, 129
- Unreife zellige Panmyelose, 174
- Unstable, new haemoglobin haemoglobin Buenos Aires, β 85 (F 1) phe-ser, 357
- Uridine (^3H uridine) v Asparaginase
- Varia, 364
- Vergleichende Hamatologie, v Eosinophile
- Vincristine, treatment of terminal metamorphosis of chronic granulocytic leukaemia with corticosteroids and vincristine, 1
- Virusinfekt (Rubeolen), neuartige Einschlüsse im Ergastoplasma peripherer Lymphozyten bei Virusinfekt, 245
- Viskositätsuntersuchungen mit Fibrinogenspaltprodukten, 75
- VON WILLEBRAND's disease, v Australia antigen
- WALDENSTRÖM's disease, v Pyroglobulinaemia
- WILLEBRAND, v VON WILLEBRAND
- Zellkulturen, v Asparaginase, Rat leucocyte cultures
- Zytophotometrie, v Erythropoese
- Zyto , v Cyto

Index autorum ad Vol. 50

(B) = Book reviews - Buchbesprechungen - Livres nouveaux

- Aiuti, F., Lacava, V., Fiorilli, M., and
 Ciarla, Maria Vera, 275
 Altay, Ç., v. Özsoylu, S
 Altay, Ç., v. Yamak, B
 Angelopoulos, B., v. Fertakis, A
 Asamer, H., v. Huhn, D
 Atencio, R. Perez, v. Uriarte, A
 Baccarani, M., v. Tura, S
 Baccarani, M., v. Zaccaria, A
 Balestrieri, G., v. Invernizzi, F
 Barbui, T., Battista, R., and Dini, E., 25
 Bassi, F., v. Buscarini, L
 Battista, R., v. Barbui, T
 Berelian, F., v. Rahbar, S
 Brand, E. T., v. Meuret, G
 Brittinger, G., v. Douglas, S. D
 Broström, J., 143
 Brunet, R., v. Hoern, B
 Brunetti, A., v. Girolami, A
 Bungert, H.-J., v. Leonhardt, H
 Buscarini, L., and Bassi, F., 223
 Castrén, O., v. Markkanen, T
 Cattaneo, R., v. Invernizzi, F
 Cheng Go Suat, v. Suat Cheng Go
 Chrobak, L., v. Mulla, N
 Ciarla, Maria Vera, v. Aiuti, F
 Cohnen, G., v. Douglas, S. D
 Colmerauer, M. E. M., v. Giraud Conesa
 L. C.
 Colombo, B., v. Uriarte, A
 Conesa, L. C. Giraud, v. Giraud Conesa
 L. C.
 Constable, T. B., v. Dunn, C. D. R
 Cserháti, I., and Tóth, S., 168
 Damasio, E. E., v. Marmont, A. M
 Daneshmand, P., v. Rahbar, S.
 de Weinstein, B. I., White, J. M., Wiltshire,
 B. G., and Lehmann, H., 357
 di San Secondo, Rosso V., v. Rosso di San
 Secondo, V
 Dini, E., v. Barbui, T
 Djaldetti, M., Rubinstein, Julia, Lewinski,
 U., and Mandel, M., 44
 Dosne Pasqualini, C., v. Giraud Conesa,
 L. C.
 Douglas, S. D., Cohnen, G., König, E., and
 Brittinger, G., 129
 Douwes, F. R., v. Hauswaldt, Ch
 Dunn, C. D. R., and Constable, T. B., 9
 Essien, E. M., Smith, J. A., and Francis,
 T. I., 293
 Falter, Maria L., Robinson, Margaret G.
 Ok Soon Kim Suat Cheng Go, and
 Taubkin, S. P., 154
 Ferran, C., v. Rozman C
 Fertakis, A., Panitsas, G., and Angelopou
 los, B., 149

- Fioretti, D., v. Girolami, A.
 Fiorilli, M., v. Ajuti, F.
 Fischer, R., v. Schaefer, H. E.
 Francis, T. I., v. Essien, E. M.
 Fudenberg, H. H., Pink, J. R. L., Sites,
 D. P., and Wang, A.-C., 191 (B)
 Gabutti, Vilma, v. Pagliardi, G. L.
 Gavosto, F., v. Pagliardi, G. L.
 Giraudo Conesa, L. C., Rumi, L., Colmer-
 auer, M. E. M., and Dosne Pasquahiti,
 C., 162
 Girolami, A., Brunetti, A., Fioretti, D.,
 and Gravina, E., 116
 Girolami, A., Scorza, P., Brunetti, A.,
 Morgagni, C., and Santini, G., 228
 Go Suat Cheng, v. Suat Cheng Go
 Gomperts, E. D., Kew, M. C., and Katz, J.,
 249
 Gonzalez, E., v. Rozman, C.
 Gravina, E., v. Girolami, A.
 Gross, R., und van de Loo, J. (Editors)
 64 (B)
 Hahn, E., v. Muller, D.
 Hauswaldt, Ch., Dourwes, F.-R., Ziesemer,
 G., und Rahlf, G., 36
 Hein, K., und Köhner, U., 217
 Hiçsönmez, G., v. Özsoylu, S.
 Hiçsönmez, H., v. Yamac, B.
 Himanen, P., v. Markkanen, T.
 Höcker, P., v. Rainer, H.
 Hoerni, B., Brunet, R., et Hoerni Simon,
 G., 213
 Hoerni Simon, G., v. Hoerni, B.
 Hübner, H., v. Schaefer, H. E.
 Huhn, D., und Asamer, H., 245
 Invernizzi, F., Cattaneo, R., Rosso di San
 Secondo, V., Balestrieri, G., and Zanusi,
 C., 65
 Kalkoff, K. W., v. Meuret, G.
 Katz, J., v. Gomperts, E. D.
 Kelemen, E., 19
 Kew, M. C., v. Gomperts, E. D.
 Kim Ok Soon v. Ok Soon Kim
 Kissling, M., and Speck, B.
 König, E., v. Douglas, S. D.
 Köhner, U., v. Hein, K.
 Lacava, V., v. Ajuti, F.
 Lauterbach, H., v. Müller, D.
 Lehmann, H., v. de Weinstein, B. I.
 Lemonen, Ellen A., 269
 Leonhardt, H., und Bungert, H.-J., 75
 Lewinski, U., v. Djaldetti, M.
 Lewis, R. A. (and Opare Mante, A.), 105
 Littlewood, Valerie v. Riches, A. C.
 Loo, J. van de, v. van de Loo, J.
 Lowka, K., v. Meuret, G.
 Mandel, M., v. Djaldetti, M.
 Markkanen, T., Himanen, P., Pajula, R.-L.,
 and Molnár, G., 284
 Markkanen, T., Himanen, P., Pajula, R. L.,
 Ruponen, S., and Castren, O., 85
 Marmont, A. M., and Damasio, E. E., 1
 Meister, H., und Trux, F., 174
 Meuret, G., Lowka, K., Brand, E. T., and
 Kalkoff, K. W., 329
 Molnár, G., v. Markkanen, T.
 Moores, Phyllis, 299
 Morgagni, C., v. Girolami, A.
 Moser, K., v. Rainer, H.
 Müller, D., Lauterbach, H., Pouillon,
 H. G., und Hahn, E., 340
 Mulla, N., and Chrobak, L., 112
 Nowzari, G., v. Rahbar, S.
 Ok Soon Kim, v. Falter, Maria L.
 Onel, D., Ulutun, S. B., and Ulutun, O. N.,
 238
 Opare Mante, A., v. Lewis, R. A.
 Özer, F. T., v. Özsoylu, S.
 Özsoylu, S., Hiçsönmez, G., and Altay, Ç.,
 184
 Özsoylu, S., and Özer, F. L., 305
 Özsoylu, S., v. Yamac, B.
 Pagliardi, G. L., Gabutti, Vilma, and
 Gavosto, F., 257
 Pajula, R. L., v. Markkanen, T.

Panitsas, G, v Fertakis, A

Pasqualini, C Dosne, v Dosne Pasqualini, C

Perez Atencio, R, v Atencio, R Perez
Pink, J R L., v Fudenberg, H H
Pittermann, E, v Rainer, H
Pouillon, H G, v Müller, D

Rahbar, S, Berelian, F, Nowzari, G, and
Daneshmand, P, 30

Rahlf, G, v Hauswaldt, Ch

Rainer, H, Höcker, P, Pittermann, E, und
Moser, K, 200

Ribas Mundo, M, v Rozman C

Ricci P, v Zaccaria, A

Riches, A C, Sharp, J G, Littlewood,
Valerie and Thomas, D B, 50

Robinson Margaret G, v Falter, Maria L.

Rosso di San Secondo, V, v Invernizzi, F

Rozman, C, Triginer, J, Ribas-Mundo,
M, Ferran C, Visa J, and Gonzales,
E, 321

Rubinstein, Iulia, v Djaldetti M

Rumi, L, v Giraudo Conesa, L C

Ruponen S, v Markkanen, T

San Secondo di, Rosso V, v Rosso di San
Secondo, V

Santini, G, v Girolami, A

Say, B, v Yamak, B

Schaefer, H E., Hübner, G, und Fischer
R, 92

Scorza, P, v Girolami, A

Sharp, J G, v Riches A. C

Smith, J A., v Essien, E. M

Soon Kim Ok, v Ok Soon Kim

Speck B, 64 (B), 192 (B)

Speck, B, and Kissling M, 193

Stites, D P, v Fudenberg, H H

Suat Cheng Go, v Falter, Maria L.

Talb-Cazal, E, 56

Taubkin, S P, v Falter, Maria L.

Thomas, D B, v Riches, A C

Tóth, S, v Cserháti, I

Triginer, J, v Rozman, C

Trux, F v Meister, H

Tura, S, and Baccarani, M (Editores),
64 (B)

Tura, S, v Zaccaria, A.

Ulutin, O N, v Onel, D

Ulutin, S B, v Onel, D

Uriarte, A, Atencio, R Perez, and Colom-
bo, B, 315

van de Loo, J v Gross, R

Visa, J, v Rozman, C

Vischer, T L, 191 (B)

Wang, A -C, v Fudenberg H H

Weinstein, B I de, v de Weinstein, B I

White, J M, v de Weinstein, B I

Wiltshire, B G, v de Weinstein, B I

Yamak, B, Özsoyiu, S, Altay, Ç., Hıçsön-
mez, G, and Say, B, 124

Zaccaria, A, Ricci, P, Baccarani, M, and
Tura S, 350

Zanussi C v Invernizzi, F

Ziesenfer G, v Hauswaldt Ch.



Panitsas, G , v Fertakis, A

Pasqualini C Dosne, v Dosne Pasqualini C

Perez Atencio, R , v Atencio, R Perez

Pink, J R L, v Fudenberg, H H

Pittermann, E, v Rainer, H

Pouillon, H G , v Müller, D

Rahbar, S, Berelian, F, Nowzari, G , and Daneshmand, P, 30

Rahlf, G , v Hauswaldt, Ch

Rainer, H, Höcker, P, Pittermann, E, und Moser, K, 200

Ribas-Mundo, M, v Rozman C

Ricci P, v Zaccaria, A

Riches, A C, Sharp, J G, Littlewood, Valerie, and Thomas, D B, 50

Robinson, Margaret G, v Falter, Maria L.

Rosso di San Secondo, V, v Invernizzi, F

Rozman C, Triginer, J, Ribas Mundo, M, Ferran C, Visa J, and Gonzales, E, 321

Rubinstein, Iulia v Djaldetti, M

Rumi L, v Giraudo Conesa, L C

Ruponen, S, v Markkanen, T

San Secondo di, Rosso V, v Rosso di San Secondo, V

Santini, G, v Girolami, A

Say, B, v Yamak B

Schaefer, H E., Hübner, G, und Fischer, R, 92

Scorza, P, v Girolami, A

Sharp, J G, v Riches A. C

Smith, J A, v Essien, E M

Soon Kim Ok v Ok Soon Kim

Speck, B, 64 (B), 192 (B)

Speck, B, and Kissling M, 193

Sittes, D P, v Fudenberg, H H

Suat Cheng Go, v Falter, Maria L.

Talb-Cazal, E, 56

Taubkin, S P, v Falter, Maria L.

Thomas, D B, v Riches, A C.

Tóth, S, v Cserháti, I

Triginer, J, v Rozman, C

Trux, F v Meister, H

Tura, S, and Baccarani, M (Editores), 64 (B)

Tura, S, v Zaccaria, A

Ulutin, O N, v Onel, D

Ulutin, S B, v Onel, D

Uriarte, A, Atencio, R Perez, and Colombo B, 315

van de Loo, J v Gross, R

Visa, J, v Rozman, C

Vischer, T L, 191 (B)

Wang, A-C, v Fudenberg H H

Weinstein, B I de, v de Weinstein, B I

White, J M, v de Weinstein, B I

Wiltshire, B G, v de Weinstein, B I

Yamak, B, Özsoylu, S, Altay, Ç, Hıçsönmez, G, and Say, B, 124

Zaccaria, A, Ricci, P, Baccarani, M, and Tura, S, 350

Zanussi, C, v Invernizzi, F

Ziesemer, G, v Hauswaldt Ch.

